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DIFFERENCE IN RESPONSE OF THE CAPON COMB TO INTRAPERITONEALLY AND SUBCUTANEOUSLY INJECTED TESTOSTERONE PROPIONATE[1]

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BISKIND (1) showed that an ovary placed in the spleen of a spayed rat becomes tumorous. He suggested that the tumor developed as a result of hepatic removal of the graft-secreted hormone. Later it was shown (Bernstorf (2)) that androgen secreted by the intrasplenic testis graft in the capon was not completely removed by the liver. That hepatic removal of hormone is not complete was confirmed by Werthessen and Field (3) with the female rat and by Bernstorf (4) with the female mouse. The incomplete removal of graft-produced hormone was also observed by Kullander (5) in rats given an ovarian graft at three weeks of age. Aqueous solutions of estrogens were given to patients by Evans, *et al.* (6) and within 60 minutes blood samples were taken simultaneously from the femoral artery and hepatic vein. The estrogen content of the hepatic vein was in each case lower than that in the femoral artery.

The experiments cited indicate that the liver removed both androgen and estrogen from the circulation under a variety of experimental conditions. Very little is known, however, about the manner in which the "inactivating" system responds to various blood concentrations of hormone under long-term and physiological conditions. Furthermore, it is very difficult to explain the behavior of the intraportal-area gonad graft because we do not know how much hormone is produced by the graft or what portion of this hormone is "inactivated" by the liver. The following experiment was designed to place hormone in a position where it was hoped it would be absorbed into the portal system and carried to the liver, and to place the hormone in such a manner that the effects of a wide range of well-

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controlled dosages could be studied over a relatively long period of time. It was hoped that this study would contribute to our understanding of the process of tumorgenesis in the ovarian and testis grafts.

METHODS

Single comb White Leghorn cockerels (the Shaw hatchery, West Chester, Pa.) were delivered to the laboratory the third day after hatching and placed in cages having continuous lighting and a coarse screen floor. They were allowed Purina mash and water *ad libidum*. At twenty days of age the chicks were caponized, given a group and individual mark, given the first injection of hormone solution or oil carrier, and the body weight and comb measurements were recorded. The chicks were grouped so that usually 16 received a certain dosage; of these, half received the injection intraperitoneally, and half, subcutaneously. The groups were not separated in the cages. The hormone was testosterone propionate[2] made into a stock solution in sesame oil. The required dilutions were made from this in such a manner that each chick received 0.05 cc. of solution per day. Injections were made each day during the experimental period.

For the intraperitoneal injections the needle was inserted into the right side of the chick in such a way that the hormone was released immediately dorsomedial to the gizzard. By this method the hormone was placed in an area completely surrounded by viscera and mesentery and the possibility of contact with the body wall was reduced to a minimum. The chicks were weighed and the combs measured on the 5th, 11th, 18th, 26th, and 34th experimental days (i.e. after castration). Changes in comb size were studied both as changes in comb factor units (CFU = length in mm. × height in mm. divided by 2) and as CFU adjusted to body weight. The former method appeared the most helpful over the range of hormone dosages employed. Other chicks were similarly used in a study with TP pellets. The TP was made into either pure pellets or mixed with cholesterol to give the desired absorption rate. The pellet was placed either in the spleen or subcutaneously, at the time of caponization.

RESULTS

When the body weight gain of the subcutaneously-treated (SC) groups was compared either with that of the intraperitoneally-treated (IP) groups or with that of the oil controls, little difference was found except in the 500 μg. dosage groups. Here the small difference between the IP and SC groups increased steadily until the 26th day when the IP value was significantly larger than the SC value. Interestingly, the value for the oil control was intermediate between that of the two hormone treated groups.

Table 1 shows the dosages of TP employed and the cumulative average increase in CFU for each group of chicks at each measurement time. These average increases were obtained by subtracting the average number of CFU for the group at experimental day zero from the average number of CFU on the day shown. It is evident that the comb size was increased both by augmented dosage and extended time. It is also seen that the comb size of animals which were given TP subcutaneously is larger than that of ani-

[2] Crystalline testosterone propionate was supplied through the courtesy of Dr. Normal L. Heminway of the Schering Corporation.

mals given TP intraperitoneally. This is illustrated clearly in two ways. Comparison of the subcutaneous group values with the intraperitoneal group values reveals that they are significantly different, except at the earlier days. A significant difference in response between the subcutaneous and intraperitoneal animals was obtained with a 20 μg. dosage at 5 days. By the 11th day a significant difference was obtained at the 5 μg. level. The greater efficacy of the subcutaneously given TP over that given interperitoneally is further shown when the oil control group is considered. At 5 days 5 μg. given SC as compared with 100 μg. given IP was required to significantly increase the comb size over that of the oil-injected control. By the 26th day the required IP dosage was reduced to 20 μg.

TABLE 1. CUMULATIVE AVERAGE INCREASE IN COMB FACTOR UNITS (CFU) FOR SUBCUTANEOUS (SC) AND INTRAPERITONEAL (IP) ROUTES OF TESTOSTERONE PROPIONATE ADMINISTRATION

μg./day	0 to 5 days		0 to 11 days		0 to 18 days		0 to 26 days		0 to 34 days	
	SC	IP	SC	IP	SC	IP	SC	IP	SC	IP
Oil control	17.9		51.2		86.1		127.1		(171.5)*	
1.25	15.6	22.2	55.7	48.1	84.0	71.1	139.0	107.2	239.0	201.3
2.5	21.6	24.5	54.2	38.3	92.3	89.9	144.4	122.9	237.6	194.3
5.0	*33.8*	17.2	(*92.7*)	(45.6)	*174.7*	89.7	*288.1*	145.3	419.8	226.2
10.0	32.2	24.0	98.2	60.7	(175.2)	(102.3)	(282.5)	(166.0)	(440.7)	(246.6)
20.0	(60.0)	(24.8)	160.2	61.1	331.6	116.2	570.8	*184.3*	821.7	293.9
50.0	55.6	25.9	165.9	65.2	379.8	*146.0*	701.6	259.1	—	—
75.0	75.9	26.3	244.5	76.7	529.7	166.5	853.9	247.0	1252.3—	378.1
100.0	90.6	*33.0*	265.8	*102.7*	577.6	172.0	992.0	273.0	1397.3—	428.7
150.0	92.9	29.9	296.2	80.3	627.4	171.5	990.0	296.8	1375.2—	479.3
200.0	87.9	46.5	313.3	112.4	687.7	239.3	1181.8—	366.1	1610.5—	594.2
250.0	94.6	47.1	326.6	122.2	693.7	287.2	1160.1—	526.2	1553.4—	769.4
500.0	121.1	47.1	392.8	159.7	816.8	452.9	1293.3—	799.7	1698.2—	1200.7

( ) Lowest dosage level giving T value significant at 1% level when response of subcutaneous route is compared with intraperitoneal route.
Italicized figures indicate lowest dosage level giving T value significant at 1% level when response to either subcutaneous or intraperitoneal route is compared with the oil control.
* Value gained by extrapolation.

Figure 1 shows that when the comb response of the SC groups as measured on any day is plotted against log-dose, the points arrange themselves along a straight line. The lack of difference between the response to 1.25 and 2.5 μg. may indicate that the lower dosage was below the limit of sensitivity for the experimental design. When the response to IP administered hormone is plotted against log-dose (Fig. 2), the lines appear quite different than the lines of Figure 1. At 5 days there is a faint suggestion that the otherwise straight line is bent upwards at the higher-dosage end. By 11 days this suggestion of a break is stronger and by the 18th and 26th days the break seems definite. The difference in response to SC and IP injections is emphasized when the two response lines for the 26th day are plotted together (Fig. 3). The standard deviation of the values is also shown. The comb response (CFU at 11 days) obtained with intrasplenic and subcutaneous pellets (Figs. 1 & 2) is very similar to that obtained with IP and SC injected hormone.

More light is thrown upon the changing relationship between the IP and SC lines by Figure 4, line one. Here the hormonal part of the IP period-

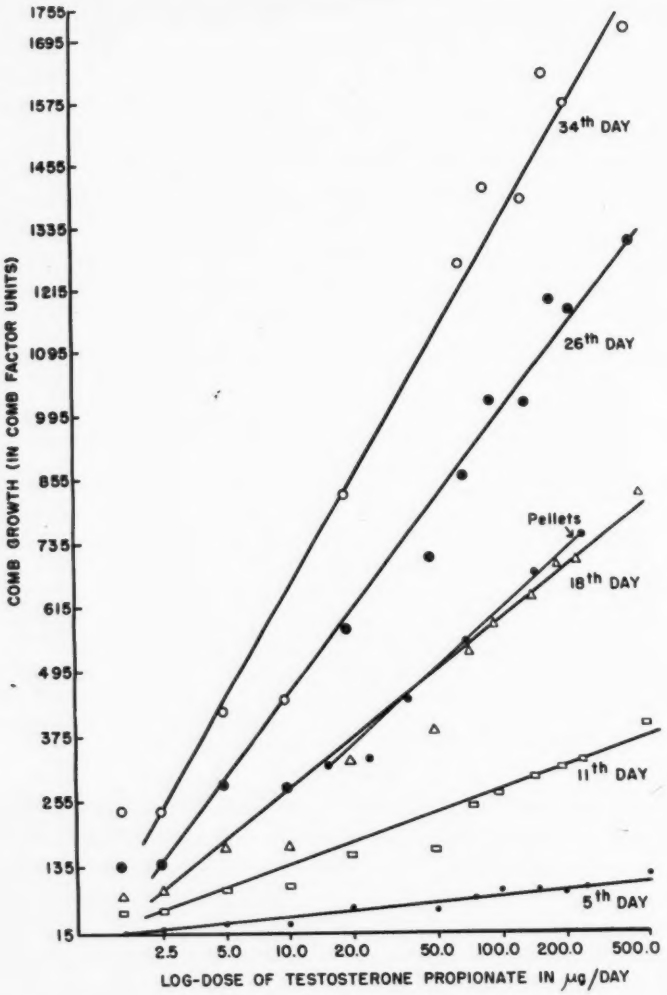

FIG. 1. Comb growth in response to testosterone propionate administered subcutaneously as injections in oil or as pellets, plotted as response vs log-dose. Each line shows the amount of comb growth from experimental day zero to the day indicated.

increase value is plotted as a percentage of the corresponding SC value. It is seen that the percentage declines rapidly with dosage increments up to approximately 20 μg., at which dosage the line flattens out and later begins to climb again. In the steeply descending portion of the curve the IP hormone shows very little effect, while the SC response increases. The line flattens as the increasing effectiveness becomes more similar for the two

routes. Beyond 150 μg. the increase in effectiveness in the IP groups is more rapid than that in the SC groups. In Figure 5 the percentage of activity loss is expressed as calculated micrograms-of-TP-loss for each IP dosage. With increments of dosage, in the lower dosage range, the amount

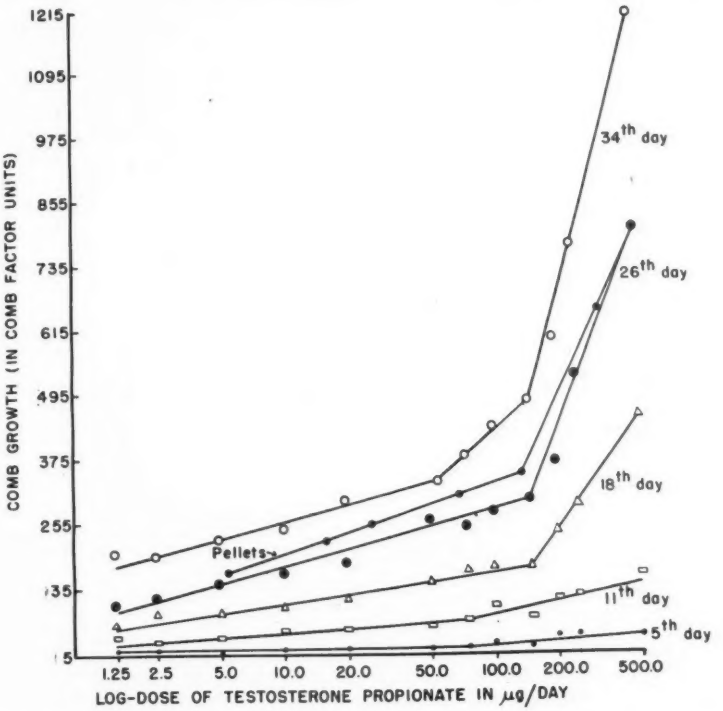

FIG. 2. Comb growth in response to testosterone propionate administered by intraperitoneal injections or intrasplenic pellets, plotted as response vs. log-dose. Each line shows the amount of comb growth from experimental day zero to the day indicated.

of loss increases very rapidly. With larger dosages the loss differs according to the day of measurement.

The effect of time upon the response to a given dosage may be further observed in Figure 6. With this treatment of the data comb growth appears fairly constant (i.e. a straight line) for any one group. Very little difference is seen between the lines of the oil control group and the 5 μg. IP group, but above 5 μg. the slope of the lines increases with augmented dosage.

Figure 7 shows the calculated μg. of hormone loss plotted against time. The amount of loss is small and fairly constant for the lower dosages. Even for the 100 μg. dosage the line remains quite flat. Beginning with 150 μg. and continuing through the 250 μg. dosage there is an apparent decrease in

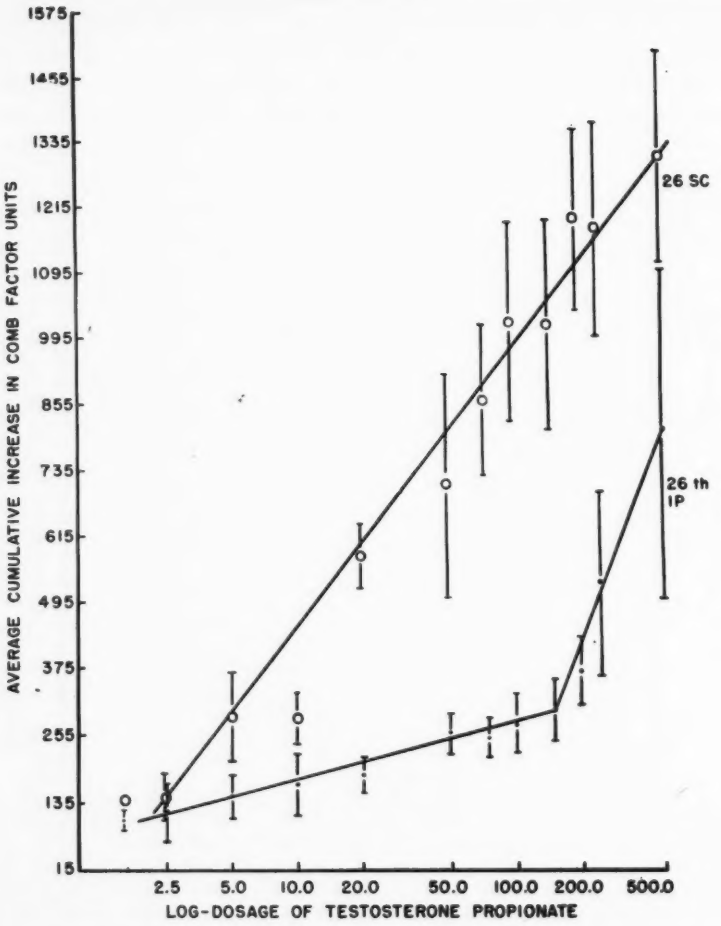

FIG. 3. Average cumulative increase in comb factor units (from Table 1) vs. log-dose for subcutaneous and intraperitoneal routes for the 18th day. Vertical lines indicate standard deviation for N−1 degrees of freedom.

the amount of activity loss. The 500 μg. line shows this trend greatly exaggerated. Considered simultaneously, Figures 7 and 6 show that the amount of activity loss is decreased both with time and with an increase of dosage above the 150 μg. level.

Figure 8 shows the daily hormone-induced comb size increase plotted against time. The daily rate of increase in CFU appears fairly constant for the 5 and 75 μg. IP dosages through the 26th day. However, the 500 μg. IP dosage, like the SC dosages, causes considerable increase in growth rate from day to day.

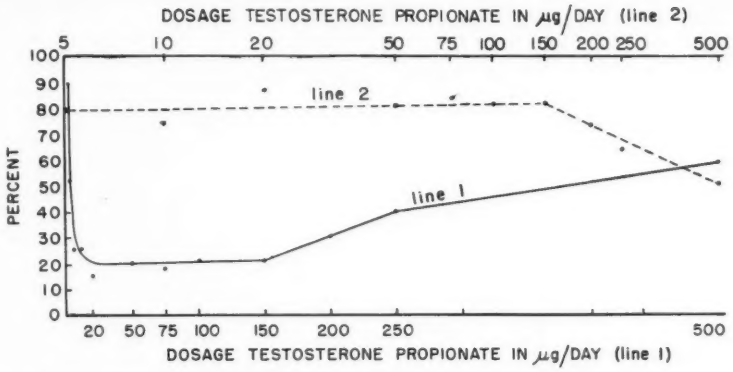

FIG. 4. Effectiveness ratio of intraperitoneal (IP) and subcutaneous (SC) hormone and the calculated per cent of activity-loss with IP-injected hormone.

Line 1: The ratio of the I P hormone-induced comb growth to the SC-induced comb growth:

$$\text{Percentage} = \frac{\text{IP average comb growth of group—capon control}}{\text{SC average comb growth of group—capon control}}$$

Line 2: The calculated per cent of hormone loss in the IP group

$$\text{Percentage} = \frac{\text{dosage} - \left[\text{dosage} \times \left(\frac{\text{IP total induced comb growth}}{\text{SC total induced comb growth}}\right)\right]}{\text{Dosage}} \times 100$$

DISCUSSION

The straight-line comb response for SC administered hormone has been shown previously by Breneman and Mason (10) for the chick and Barnes *et al.* (7) for the seminal vesicle weight in the rat. The latter work is of especial interest since it also employed 500 μg. as the highest dosage. The ineffectiveness of IP injected TP has been shown previously by Leathem (9) with the immature rat.

Some IP injected hormone may have been absorbed through the lymphatics; however, Hyde *et al.* (8) gave 17α methyl-C^{14}-testosterone in either alcohol or oil by gavage to rats having a canulated thoracic duct and found less than 1.5% of the hormone in the lymph in 24 hours. It would seem that lymphatic absorption from the peritoneal space and outer surface of the gut would be no greater than from the inner gut surface. A large part or most of the IP hormone was possibly absorbed into the portal system. This possibility is supported by the data obtained with pellets of TP placed in the spleen. When the comb response (CFU at 11 days) for both the intrasplenic and subcutaneous pellet groups is plotted against the log-dose (Figs. 1 & 2), a line is obtained which is very similar to that obtained with injected hormone. Since it is generally accepted that hormone placed in the

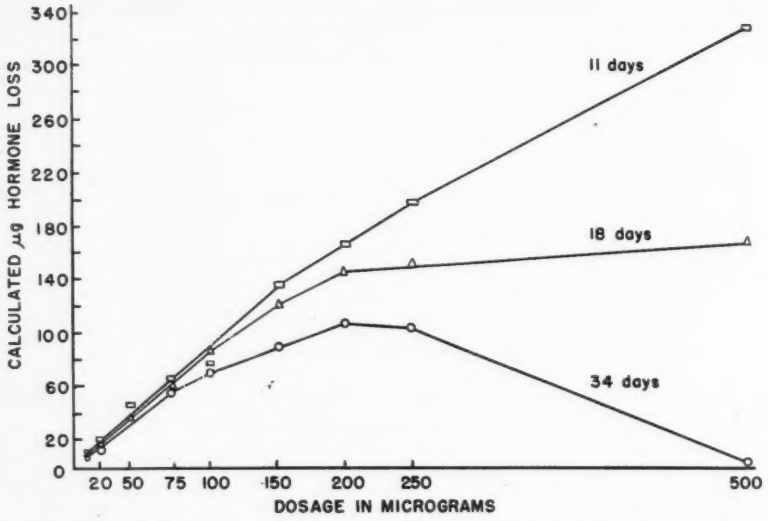

FIG. 5. Calculated micrograms of testosterone propionate loss in the intraperitoneal groups. Each line represents values obtained for the measurement period indicated; plotted as μg. loss vs. dosage.

The calculated micrograms of hormone loss =

$$\text{dosage} - \left[\text{dosage} \times \left(\frac{\text{IP hormone-induced comb growth for period}}{\text{SC hormone-induced comb growth for period}}\right)\right]$$

spleen is carried directly to the liver and that the reduction in effectiveness of intrasplenically administered hormone is due to processes within the liver, it seems highly suggestive that when the loss of hormone effectiveness is quite similar for the intrasplenic and IP routes that the cause may also be the same.

Of the possible explanations for the change in effectiveness of a given dosage of IP hormone (Fig. 2) the following appears most likely. The activity of the hormone-"inactivating" liver enzyme system(s) is increased with each increment of dosage up to approximately 150 μg., but from here on the enzyme system may be unable to keep pace with the increasing amounts of hormone and therefore increments of dosage above this level become increasingly more effective.

When the intraperitoneal response is compared with the subcutaneous response, as in Figure 4, line 1, one sees that the relationship changes greatly with changes in dosage. The intraperitoneal response represents nearly 90% of the subcutaneous response at the 1.25 μg. dosage level, but only 20% for the intermediate dosages. Because of individual variation, unfortunately nothing can be determined about the amount of TP ac-

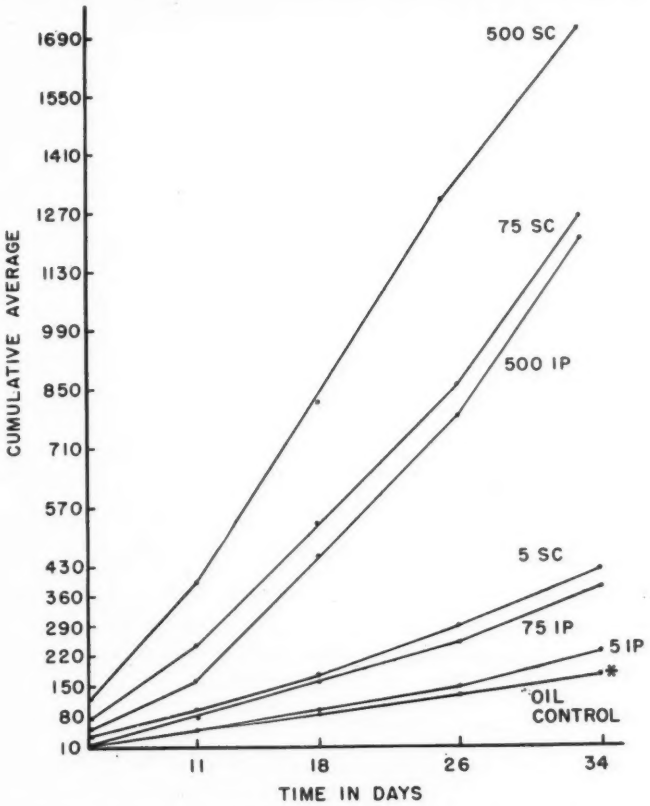

FIG. 6. Cumulative average increase in comb factor units (Table 1) vs. time in days. The line for the oil control and the 5, 75, and 500 μg. dosage levels for both the intraperitoneal and subcutaneous routes are shown. Each line represents the dosage level and route of administration as indicated. * Point gained by extrapolation.

tivity lost in the two lowest dosage groups. Comparison of Figure 4, line 2 with Figure 5 shows that the amount of loss is not constant over any portion of the dosage range. Rather, it appears that a remarkably constant *percentage* is lost over a wide dosage range (5 to 150 μg.). This is of especial interest since the 5, 10 and 20 μg. dosages fall within the physiological limits for chicks of this age, as shown by Breneman and Mason (10). We may theorize that: 1. Most of the hormone absorbed through the peritoneum from injections as described is picked up by the portal system and carried to the liver. 2. The difference in response to TP administered subcutaneously and intraperitoneally reflects the loss of hormone activity through hepatic "inactivation," although a difference in speed of absorp-

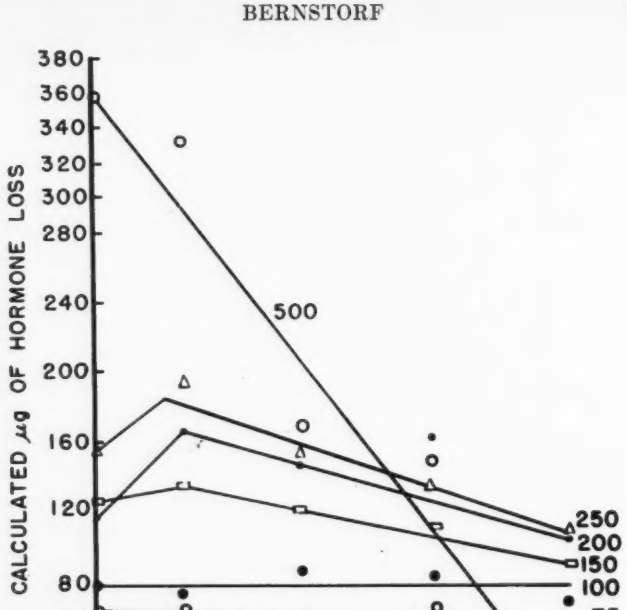

FIG. 7. Effect of time upon the calculated hormone loss in micrograms. See Figure 5 for explanation of derivation. Plotted as calculated μg. loss vs. time; each line represents a dosage level.

tion may also be involved. 3. A fairly constant percentage (perhaps 80%) of the TP within a dosage range of 5 to 150 μg. is "inactivated" as it is brought to the liver.

It was hoped that this study would contribute to our understanding of the process of tumorgenesis in the ovarian and testis grafts. If during the first few days after a graft was placed, 80% of its hormone secretion were removed by the liver, very little would enter the systemic circulation to exert a regulatory influence upon the pituitary. On the basis of the calculated TP loss plotted in Figure 5 we may theorize that the activity loss is increased more rapidly than the secretory activity and that the pituitary imbalance is further exaggerated. This theory seems reasonable in view of the rapid increase in graft size (Bernstorf (4), and others), the small size of the uterus and vagina in the graft-bearing animal, and the inability to stimulate the graft to further size increase by relatively large dosages of gonadotrophin(s) (Bernstorf (11)).

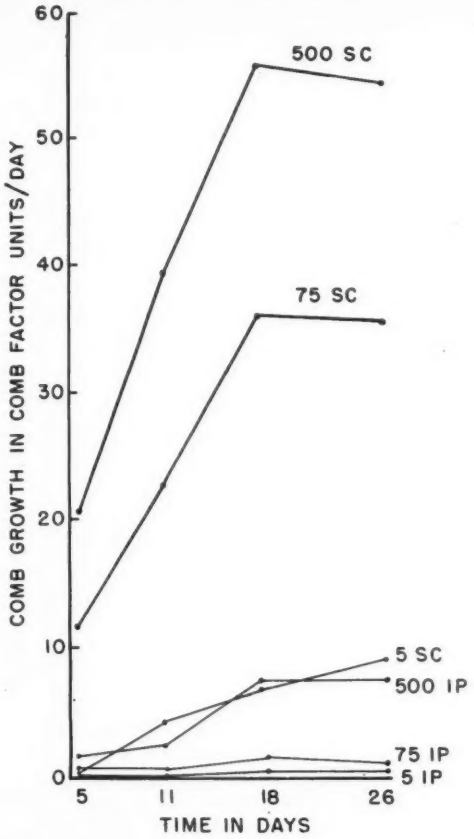

FIG. 8. Rate of comb growth in response to 3 dosage levels of testosterone propionate when given intraperitoneally (IP) and subcutaneously (SC). The daily hormone-induced increase in comb factor units is plotted against time in days. Each line represents one route and dosage level as indicated.

SUMMARY

White Leghorn capons were divided into groups at 21 days-of-age and started on a series of 34 daily injections of testosterone propionate (TP) given either subcutaneously (SC) or intraperitoneally (IP). The range in dosage was from 1.25 μg. to 500 μg./day. The chicks were weighed and the combs measured at given intervals.

The hormone effect was considered in terms of comb-growth stimulating activity. The TP given SC caused a straight line comb response, but given IP there was a great loss in effectiveness up to approximately the 150 μg. dosage level. From this point the effectiveness of IP injected hormone increased rapidly with the higher dosages. The SC response was significantly

greater than the IP response except with the smaller dosages. The data are discussed in terms of the percentage of hormone activity lost with IP injection, and in terms of their relationship to the endocrine imbalance found in the castrate animal bearing a gonad graft in the portal drainage area.

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AN ELECTRON MICROSCOPIC STUDY OF THE CASTRATION CHANGES IN THE RAT PROSTATE

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REDUCTION of prostatic mass and of the size of the individual epithelial cells of the gland are known to follow castration. With the advent of techniques for thin sectioning and of the increased resolution afforded by the electron microscope, it seemed profitable to study the cells of the prostate with particular respect to changes in the fine structure following various endocrinologic alterations.

In this paper the fine structure of the prostate of the normal rat is defined as well as the changes that follow castration. No previous electron microscopic study of the prostate has been reported.

MATERIALS AND METHODS

Twenty-two 175-gram male rats were used. Four served as controls. The others were castrated by excising the testes and a segment of spermatic cord, under ether anesthesia, using clean but not sterile technique. The animals were killed in groups of three on 1, 2, 4, 8, 16, and 32 days after castration. Two of the control animals were examined at the beginning of the experiment and two at the end. The animals were killed by crushing the base of the skull and cervical vertebrae.

After the prostate was exposed, fragments one to two mm. in diameter selected from those parts of the ventral prostate most removed from the urethra, were placed immediately in chrome osmic acid fixative (1) and fixed for one to two hours at 8° C. After the tissue had fixed for 7–15 minutes the solution was poured off and fresh fixative added. The fixative consisted of 1% osmic acid in 1% aqueous potassium bichromate, buffered to pH 7.2. The tissues were then rapidly washed in distilled water and dehydrated in 70% ethanol (30 minutes, 2 changes); 95% ethanol (30 minutes, 2 changes); and absolute ethanol (30 minutes, 2 changes). Then they were placed in a mixture of equal parts of absolute alcohol and methacrylate monomer (30 minutes); pure monomer (mixture of 1 part methyl methacrylate to 7 parts of butyl methacrylate for 30 minutes); and in a solution of the monomer, catalyzed with benzoyl peroxide, for 30 minutes. Specimens were transferred to gelatine capsules containing partially polymerized methacrylate. The capsules were hardened by heating at 45° C for 24 hours.

Thin sections were cut with glass knives on a Servall Porter-Blum microtome (2). Thicker sections were cut from the same blocks and examined with the phase microscope. As the thin sections were cut, they were floated onto a bath of 20% acetone in distilled water kept on the glass knife by a paraffin dam. Before the sections were placed on grids the 20% acetone solution was removed in part and replaced by 40% acetone.

The sections were mounted on celloidin-covered copper mesh grids. Examinations were made in one of three RCA electron microscopes, models EMU-2C, 2E, and 3B.

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In the EMU-3B microscope the electrons were accelerated by 50 or 100 kv. The EMU-2C and 2E microscopes were modified by the addition of an apparatus that allowed the objective aperture to be centered from the exterior of the machine. Platinum apertures with a central bore of 1 mil were used for the objective aperture.

Photographs were taken on Kramer high-contrast lantern slide plates developed in Kodak developer D-61. The original electron micrographs were taken at magnifications of from 1000 to 10,000 and enlarged photographically as desired.

OBSERVATIONS

Morphology of the Normal Prostate

The epithelium is tall and columnar with basal nuclei (Fig. 1). The apical portion of the cytoplasmic membrane projects into the lumen with a moderate number of rather regular microvilli. No distinct interruptions in the apical investment of the cells are present. The apical endoplasmic reticulum is disposed as flattened sacs or envelopes. When the cell is sectioned in a plane through the long axis the sacs tend to be arranged as parallel structures, whereas in cross section they assume a more lamellar pattern (Fig. 4). The endoplasmic reticulum has a finely granular component in the space between the membrane-lined sacs (Fig. 3). The proportion of the apical cytoplasm occupied by sacs is relatively greater than that consumed by the intervening granular component. The sacs are lined by a thin membrane to the outer surface of which granules are closely applied (Fig. 3). Mitochondria are interspersed in the matrix between the sacs, and are particularly prominent at the most apical portion of the cell.

A microvesicular complex (Fig. 2), which could be termed a Golgi complex, is situated in the mid-region in that part of the cytoplasm between nucleus and lumen. In addition to the tiny elongated membrane-lined sacs, there are also mitochondria in the mid-portion of the cell.

That portion of the cytoplasm immediately above the nucleus is similar to the apex in having a labyrinth of sacs. But here, above the nucleus, the concentric pattern of these flattened envelopes is even more distinct (Fig. 4). A similar pattern of the endoplasmic reticulum or ergastoplasm may be observed in the infra-nuclear portion of the cell (Fig. 5).

The membranous component of the ergastoplasm is continuous with

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FIG. 1. Normal rat prostate. This electron micrograph is of a slightly tangential section showing the epithelial lining cells of a prostatic lobule. At the bottom of the picture a red blood cell lies within a capillary. The fingerprint-like whorls are the sacs of the endoplasmic reticulum. Scattered between the sacs are mitochondria, which appear as small round dark structures. The gray region (indicated by an arrow) mid-distance between cell nucleus (N) and apex is a zone of microvesicles which can be termed a Golgi complex. At the apical surface can be seen the small projections of the microvilli. At the top of the picture a fragmented nucleus lies within the lumen (L). ×6000.

FIG. 2. Normal rat prostate. This micrograph illustrates the profiles of the closely arranged elongated microvesicles found in the Golgi region. To the left of the picture can be seen the granular component of the endoplasmic reticulum. ×42,000.

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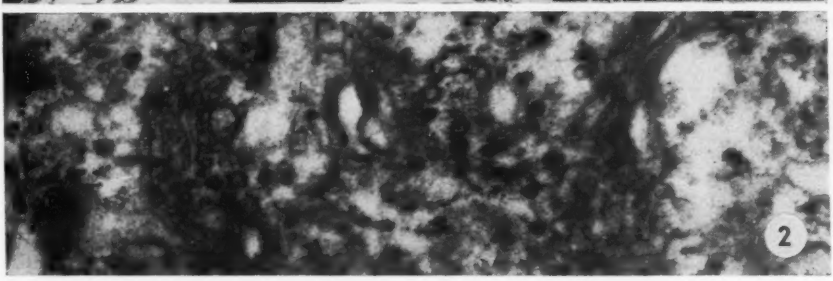
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the outer nuclear membrane. Occasionally a nuclear pore (3) can be identified (Fig. 8) where an apparent communication exists between the nucleoplasm and the granular extranuclear material. In the sections the nuclei contain finely dispersed granules.

The basal margin of the cell is a double membrane (Fig. 5), one of which continues as an uninterrupted basement membrane beneath adjacent cells. The other continues as the plasma membrane delimiting individual cell boundaries. The adjacent plasma membranes constitute the usual double membranes between epithelial cells. Separations normally are present between these membranes thereby forming intercellular spaces (Fig. 4).

An occasional cell may have somewhat more distended ergastoplasmic sacs. In addition, scattered macrophages or polymorphonuclear leukocytes are found between epithelial cells or within the lumen.

The normal rat prostate has a relatively small amount of stroma. Within this region are collagen bundles, fibrocytes, macrophages, smooth muscle cells and blood vessels.

Changes Following Castration

The earliest post-castration alterations are seen in the apical ergastoplasmic sacs. Within 24 hours after the animals have been castrated these sacs are appreciably distended (Fig. 6). There is less material within the sacs. The apices of many cells are rounded-up in a fashion to suggest that these portions are being extruded into the lumen. A decrease in the microvilli, both as to their size and number, occurs. In addition to the changes within the sacs, opaque circular and oval zones appear within the substance of the endoplasmic reticulum. These opaque bodies are increasingly prominent in the succeeding days following castration. In some of these bodies

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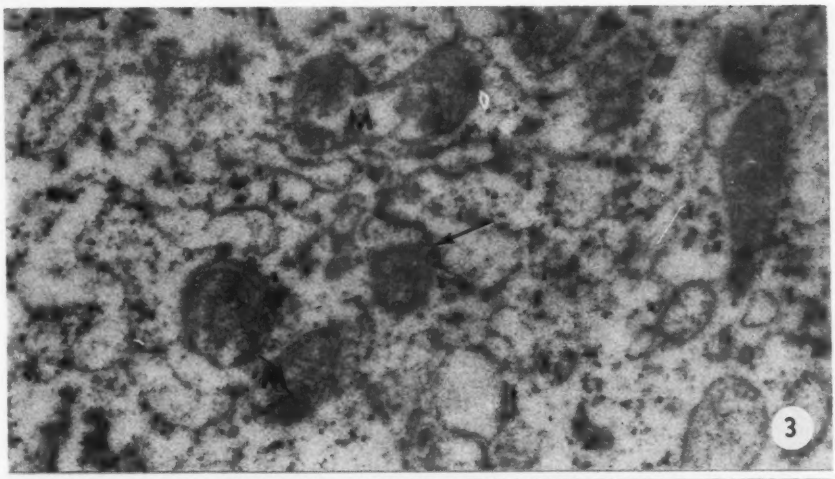
FIG. 3. Normal rat prostate. In this micrograph the pattern of the endoplasmic reticulum and the character of the mitochondria (M) in the apical portion of the cell are illustrated. The arrow points to a membrane-lined sac of the endoplasmic reticulum, which has attached to the outer surface granules. The continuity of the sacs is less obvious in this picture than in a slightly thicker section, such as the one illustrated in Figure 5. ×42,000.

FIG. 4. Normal rat prostate. The character of the lamellated ergastoplasmic sacs in the supranuclear portion of the cell is demonstrated in this electron micrograph. ×13,000.

FIG. 5. Normal rat prostate. The basal region of the cell is illustrated in this micrograph. Note that the space occupied by the sacs is roughly equal to that consumed by the granular component of the endoplasmic reticulum. The density of the granular component is roughly similar throughout the normal cell, the apparent differences between Figures 3 and 5 are a result of the differences in thickness of the sections. The arrow points to the double membrane at the bottom of the cell, consisting of the plasma membrane and the basement membrane. ×26,000.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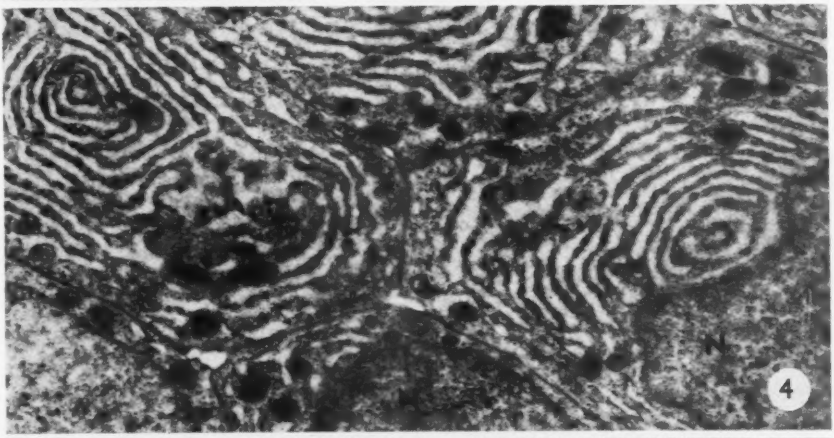
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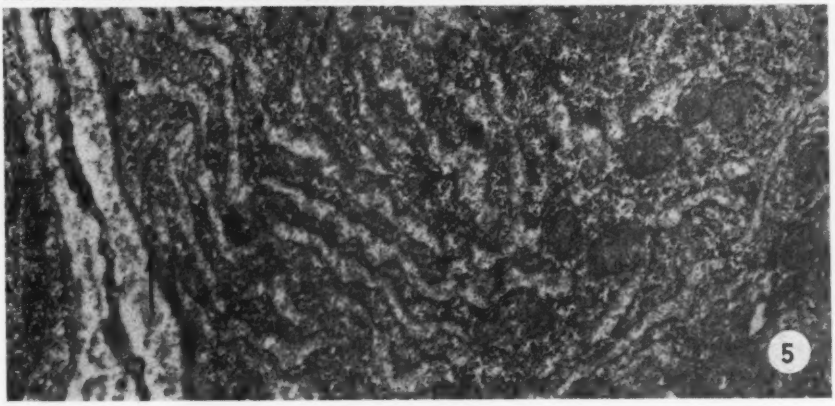
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there is a suggestion of an internal structure (Fig. 8). Others lie in close apposition to membranous sacs, apparently compressing them (Fig. 13). However on occasion these bodies may be identified lying in the intercellular spaces. These structures are prominent in the supranuclear region four and eight days after castration. Sixteen days after castration they are more numerous between cells at the basal region (Fig. 15).

By the second post-castration day the character of the cell is strikingly different (Fig. 7). Rather than distended apical sacs, the apical substance of the cell is reduced. Mitochondria are rather closely packed. Some are large. The apical endoplasmic reticulum consists principally of the granular component and only an occasional collapsed membranous complex is identified.

The collapse of the sacs of the endoplasmic reticulum is pronounced by the fourth day (Fig. 8). The superficial microvilli are as prominent as in the normal cell, and by the eighth day (Fig. 11) considerably more prominent than prior to castration. As the period following castration increased the apical endoplasmic reticulum is progressively less prominent (Fig. 15).

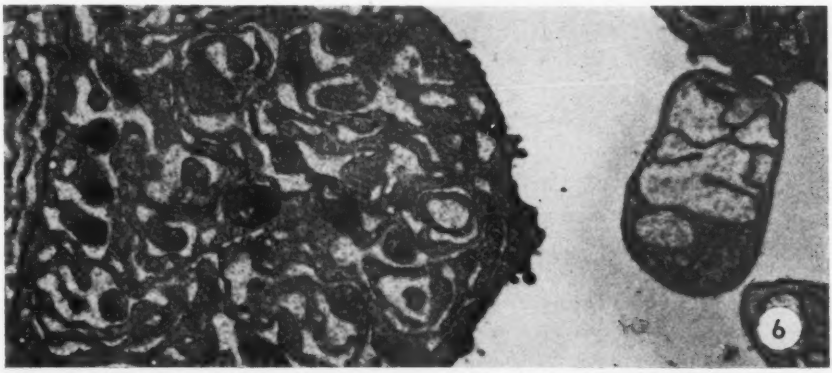
Although the changes that occur in the ergastoplasm of the entire cytoplasm are of the same type, the changes in the apex appear before those in the remainder of the cell. The size of the sacs above and below the nucleus remains relatively unchanged in sections of prostate removed two days after castration. However by four days these sacs have also collapsed, although not so completely as they do by 8 days (Fig. 14).

One day following castration there is a slight but definite increase in size of the microvesicles of the Golgi region. A further increase is apparent at two days (Fig. 7). In the specimens taken four and eight days after castration the sacs of the Golgi region are more prominent than the collapsed ergastoplasmic sacs (Figs. 8 and 11). However a categorical differentiation between the ergastoplasmic and Golgi membranes may not be critical because of the continuation of the membranous sacs with attached granules into sacs having no attached granules.

Several weeks after castration mitochondria are found dispersed within the cells with about the same intervals between them as before castration. Therefore, since the cytoplasmic volume has diminished, the total number of mitochondria in each cell appears to have been reduced. Even as early as the first post-castration day large mitochondria are present.

FIG. 6. Rat prostate, 1 day post-castration. In this micrograph the dilatated ergastoplasmic sacs in the apical region of two cells can be seen. Note particularly the rounded-up projection on the surface of the cell to the right. ×12,000.

FIG. 7. Rat prostate, 2 days post-castration. In the micrograph the supranuclear zones of several adjacent cells are seen. The mitochondria are closely spaced at the apical region. Many distended microvesicles are present in the region mid-way between apex and nucleus. ×12,000.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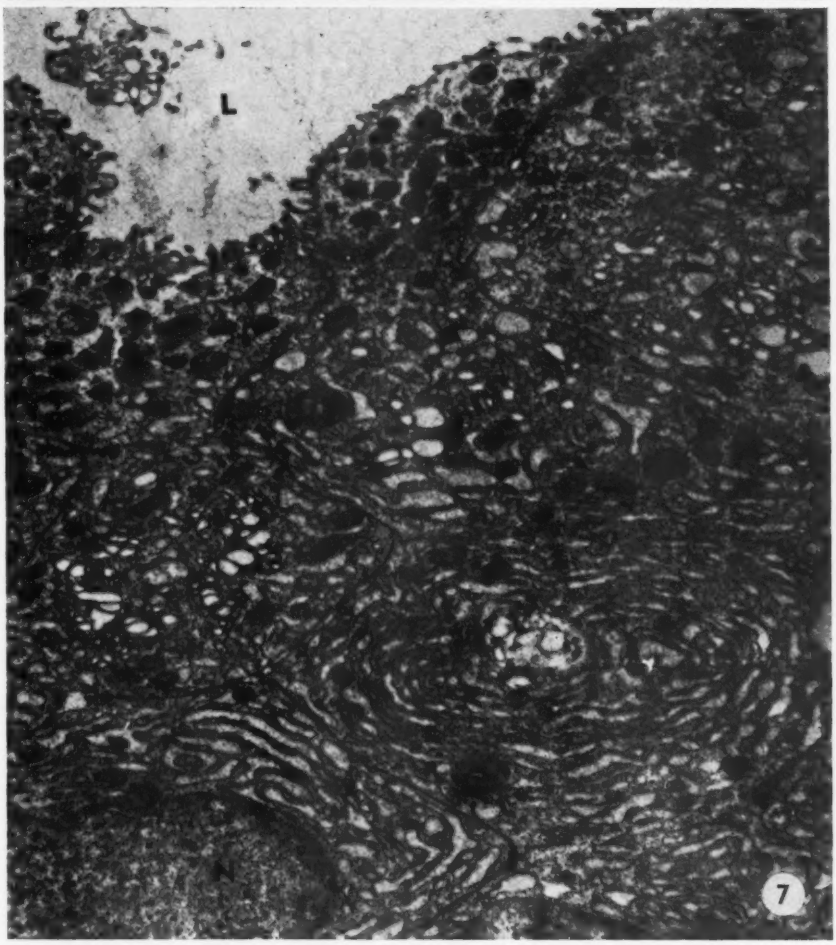
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DISCUSSION

From these studies with the electron microscope the impression might be conveyed of a stationary, almost crystal-like latticework structure of the normal prostatic epithelial cells. But the rapid rearrangement of the fine internal structures following castration is a striking demonstration of the dynamic fluid character of cells.

In previous studies of castrated animals interest has centered on the changes occurring four days or more after excision of the testes, since unequivocal evidence of cellular alteration cannot be discerned earlier by light microscopy. Indeed, at these times the cells are shrunken and changes have already occurred. However, what are these changes? What component of the cytoplasm is the one most responsible for this reduction of tissue mass?

A certain insight into this problem has been gained by rather recent histochemical studies. Brandes and Bourne (4) studied prostatic tissue of mice prepared for the examination of phosphatase and P.A.S.-positive material. It would be convenient to consider that the decrease in acid phosphatase activity that occurs following castration corresponds to the striking reduction in mitochondria in the cells of the castrate, since it is known that at least an appreciable part of the phosphatase activity of some tissues is localized to the mitochondrial fraction of the cells (5).

However, the zone roughly corresponding to the Golgi region is the site of the greatest reaction in sections prepared to demonstrate acid phosphatase. If the phosphatase is actually associated with the Golgi microvesicles, as it apparently is in some tissues (6), it is not surprising that the stained zone is less compact when these vesicles are dilated four days following castration. Later the diminution of phosphatase activity parallels the de-

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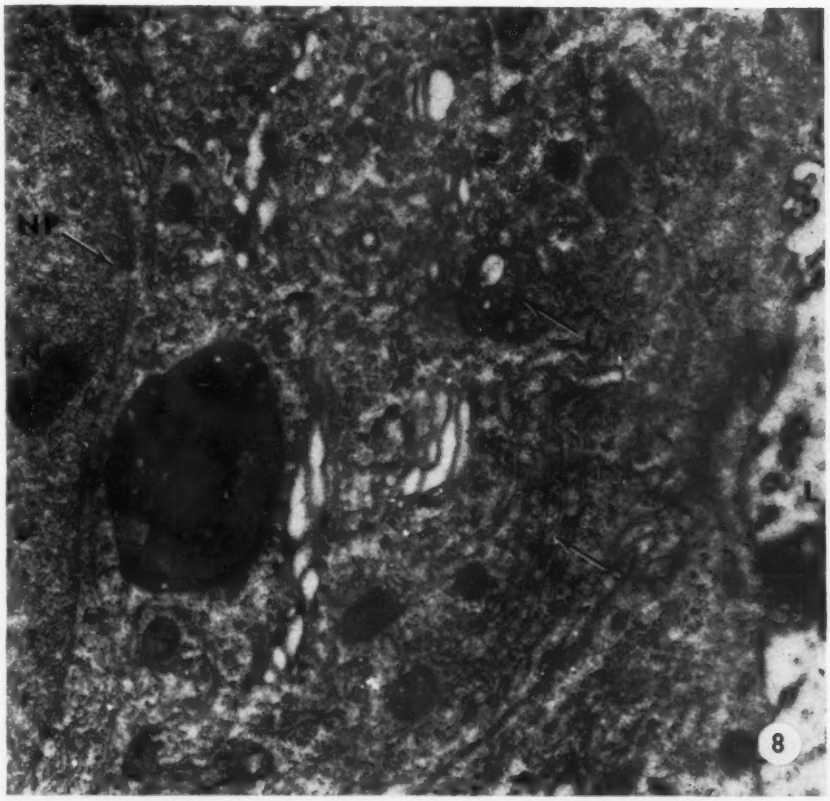
FIG. 8. Rat prostate, 4 days post-castration. Most of the changes that follow castration can be illustrated in this electron micrograph. The section includes a part of the nucleus (N) and the cytoplasm lying between the nucleus and the lumen (L).

A moderate number of microvilli cover the cell. The granular component of the endoplasmic reticulum is prominent. The arrow labelled S points to one of several collapsed ergastoplasmic sacs. In the center of the picture there are several dilatated Golgi microvesicles. Only a few mitochondria are present. Note the large mitochondrion (LM); these forms are common at this time. A large electron dense body is present at the left of the field.

Two normal structures are well illustrated in this micrograph. At the arrow labelled NP there is a nuclear pore. The communication between nucleoplasm and cytoplasm is clearly seen. The electron dense zone lying between the two cells at their apical ends probably is a terminal bar (TB). ×26,000.

FIG. 9. Rat prostate, 4 days post-castration. In this electron micrograph it is apparent that only a small amount of cytoplasm lies above the nucleus. ×10,000.

FIG. 10. Rat prostate, 4 days post-castration. The electron micrograph illustrates collapsed sacs of the endoplasmic reticulum lying adjacent to the nucleus (N). ×39,000.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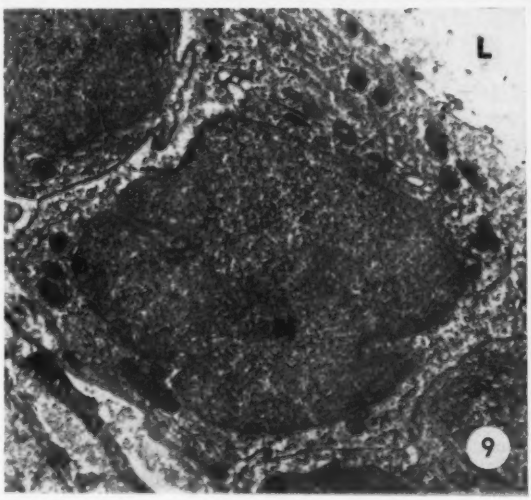
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crease in size of the Golgi complex as seen with the electron microscope.

If only one change had occurred by the end of four days, it might be less difficult to correlate histochemically localized zones with the fine structure visualized with the aid of the electron microscope. However at that time not only has there been a dramatic collapse of the envelopes of the endoplasmic reticulum, but mitochondria have been reduced in number, the granular component of the ergastoplasm has become relatively more prominent, the sacs of the Golgi region have become distended and dense bodies have appeared in the cytoplasm.

The reduction of the P.A.S.-positive component of the cells may represent the histochemical correlation of the collapsed and empty sacs of the endoplasmic reticulum. However the slight changes observed in histochemical preparations four days post-castration are disappointingly meager findings to relate to the drastically altered cells as seen with the electron microscope.

In this study an important consideration was that cells examined by electron microscopy may have apparent distention of sacs as a result of fixation artifact. Particularly difficult to interpret with certainty were those alterations found one day following castration. Several factors taken together made it difficult to believe the changes were only the result of artifact, viz. apical sacs were found consistently distended in the different samples and different animals, the remainder of the ergastoplasmic sacs and the Golgi sacs were not distended, nuclear fixation was good, and the reduction in apical mass by the second post-castration day suggested that some profound alteration must have occurred in that part of the cell.

A discussion of structural representation of secretory activity must concern itself with both rate of production and rate of liberation of secretions. The degree of dilatation of the sacs of the endoplasmic reticulum seems to be related to the degree of secretory activity of the cells. On the

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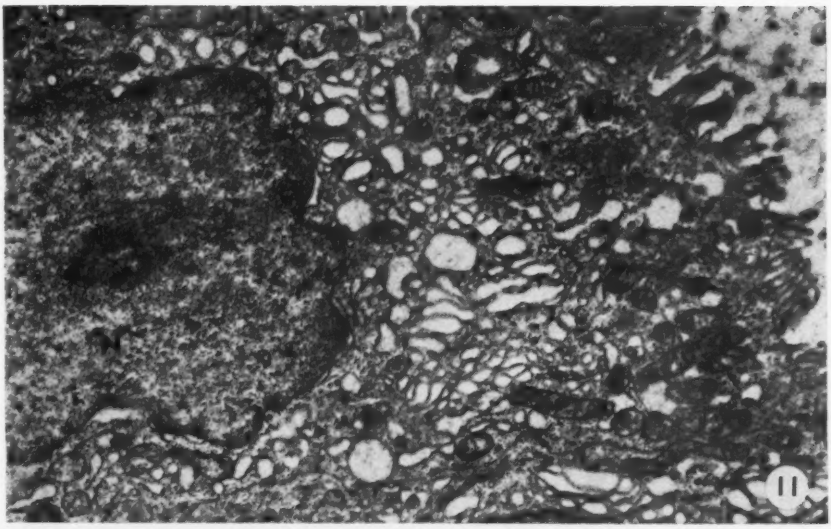
FIG. 11. Rat prostate, 8 days post-castration. In this micrograph almost an entire cell can be visualized. At the right of the field are the numerous apical microvilli. Numerous distended Golgi microvesicles lie in the region above the nucleus (N). There are scattered mitochondria, some of which are large. ×14,000.

FIG. 12. Rat prostate, 8 days post-castration. This micrograph illustrates part of the supranuclear zone of two adjacent cells. Note the double membrane separating the two cells, which diagonally traverses the picture. The granular character of the endoplasmic reticulum is prominent. The left of the field is occupied by a Y-shaped mitochondrion. ×35,000.

FIG. 13. Rat prostate, 8 days post-castration. In this electron micrograph the dilatated sacs of the Golgi region are shown in greater detail. Note how these sacs are not lined externally by a granular component. To the right of the field is an electron dense body that appears to lie at the side of a microvesicle, compressing it. To the left of the field there is an S-shaped mitochondrion. ×35,000.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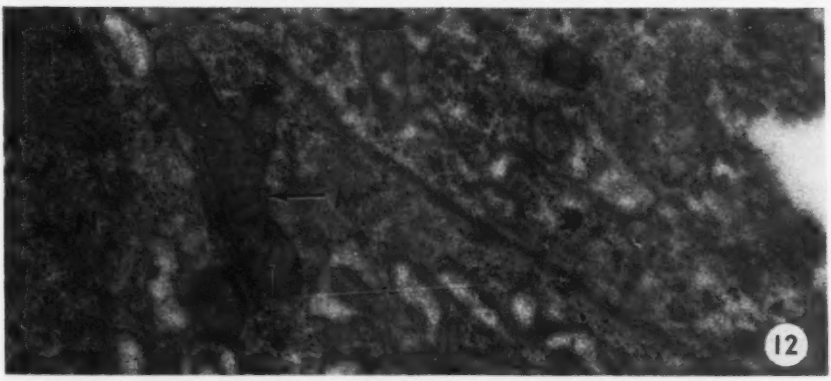
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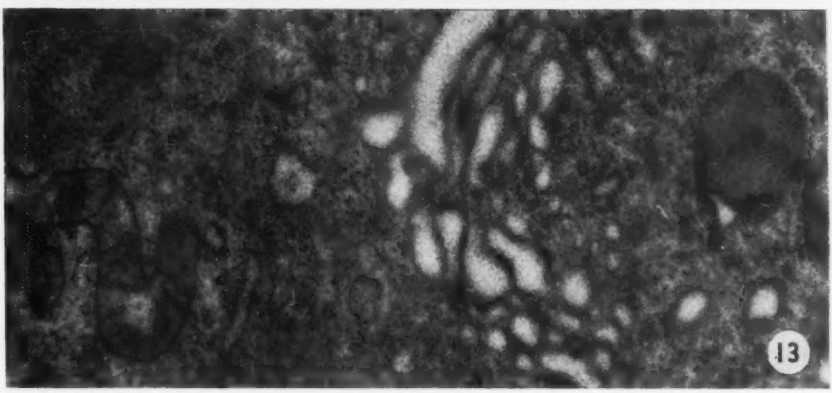
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first day following castration the ergastoplasmic sacs are distended. However, even at that time the material within the sacs is less dense, which could mean that production of secretion is already reduced and that the dilatation of the sacs is only a manifestation of a process of involution. An explanation that is favored by the author is that the function of liberation of secretions is diminished prior to a decrease in production of secretion. Such an occurrence would explain a transient dilatation of the ergastoplasmic sacs. The correlation of the secretory activity with the degree of dilatation of the ergastoplasmic sacs and the amount of material contained within them is particularly attractive when considered in relation to the striking collapse of these sacs at the time when functional activity of these cells appears to be definitely reduced, i.e. by four days.

A hypothysis as to the site of action of testosterone on the prostatic cell might be suggested, although without further data any extensive conclusion would be unwarranted. If testosterone is necessary for the release of secretory material and it functions at the plasma membrane, withdrawal of testosterone should cause temporary increased storage in the cell. Such a change is seen one day after castration, if the distended apical sacs are interpreted as sacs dilatated with secretion. But these sacs may be distended because of imbibition of extracellular fluid. If so, the phenomenon would still seem to represent the result of altered plasma membrane permeability after withdrawal of testosterone. The later gradual reduction in cytoplasmic mass following castration could be interpreted as indirect evidence that testicular hormone has a direct effect on intracellular structures. However these might be secondary changes in the cytoplasm following alteration of plasma membrane permeability. If the normal transfer of secretion across the plasma membrane is stopped or drastically reduced perhaps the impetus for the continuous elaboration of the endoplasmic reticulum is destroyed or at least considerably diminished.

In previous work on other tissues by Palade (7) the granular component of the endoplasmic reticulum has been correlated with ribonucleic acid. Its relatively small alteration would suggest that little change of this com-

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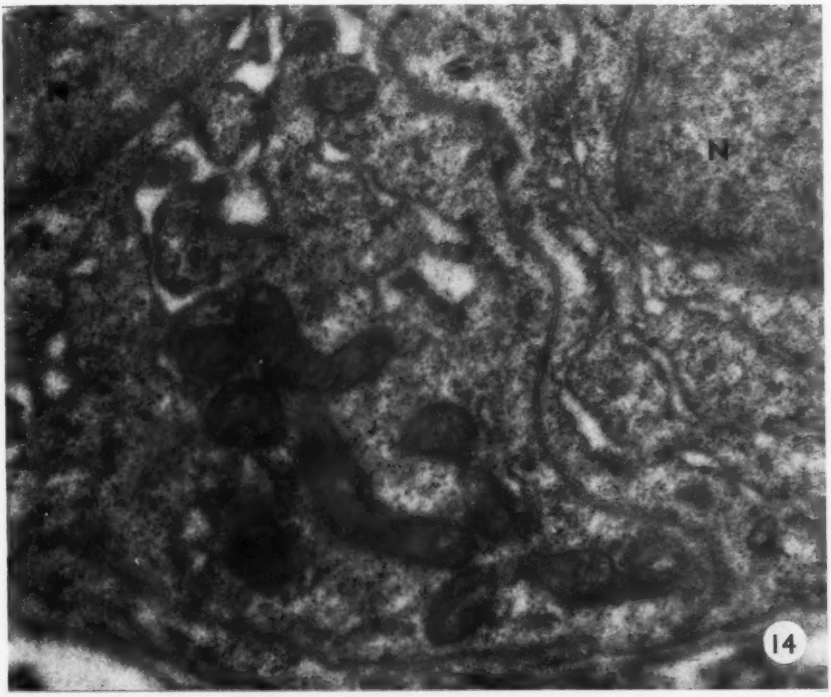
FIG. 14. Rat prostate, 8 days post-castration. The basal region of two epithelial cells is illustrated in this micrograph. At the bottom of the picture is the double membrane at the base of the cells. Note particularly the proportion of granular endoplasmic reticulum to sacs, and compare this with the ratio in the normal cell, as illustrated in Figure 5. The mitochondria (M) have a usual internal structure. ×34,000.

FIG. 15. Rat prostate, 16 days post-castration. This micrograph illustrates the wall of a gland. The lumen (L) is small. The lining cells have only a small amount of cytoplasm. Opaque bodies (OB) are present in the basal regions between adjacent epithelial cells. Several smooth muscle cells (SM) are present in the stroma at the bottom of the picture. These cells appear relatively more prominent at this time. A capillary is labelled C. ×3000.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ponent occurs until 8 days or longer after castration when the total volume of the cells becomes drastically reduced.

Moore, Price and Gallagher (8) studied the Golgi apparatus of castrates by light microscopy. They found the zone unchanged for the first four days, fragmented after 10 days, and then reduced in volume as the post-castration period lengthened. The present work suggests a temporary increase in prominence during the 8 days following castration, at least in relative prominence of the microvesicles which have been considered to be the structures corresponding to the Golgi apparatus. In the cells studied 16 and 32 days after castration the Golgi region becomes reduced in volume.

The electron dense cytoplasmic bodies which are found in the cells probably correspond to the deposits of lipochrome pigment seen with the light microscope (8). Except for the appearance of these bodies sooner after castration when studied with the electron microscope, the location and time of appearance roughly parallels the pigment granules. The electron dense bodies bear at least a superficial resemblance to structures which are commonly found in the supranuclear region of cells of the prostate from older rats (Harkin, in preparation).

The most impressive feature revealed in this study was the rapidity of the changes which develop following castration. Further studies are in progress to evaluate precisely the time when alterations initially can be detected by electron microscopy, and to see if these changes can be reversed by hormonal therapy.

SUMMARY

The epithelial cells of the rat prostate, when studied with the electron microscope, have an unusually prominent microvesicular component or Golgi complex. The endoplasmic reticulum is arranged as a labyrinth of membrane-lined sacs and as a granular component.

Within 24 hours after castration the apical ergastoplasmic sacs are swollen and there is expulsion of the superficial portion of the cytoplasm. At two days this process has progressed with dilatation of the microvesicles of the Golgi region and reduction of apical mass of the cytoplasm. By four days a massive collapse of the sacs of the endoplasmic reticulum has occurred. The later changes in the cells consist of continued reduction of cytoplasmic mass, accomplished by a reduction of all of the cytoplasmic components, but principally through collapse of the sacs of the endoplasmic reticulum.

In this study a rapidity of structural changes following endocrinologic imbalance, castration, was demonstrated by electron microscopy which had not been apparent by other morphologic techniques.

Acknowledgment

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RESTORATION OF ENZYMATIC ACTIVITY IN THE SUBMAXILLARY GLAND OF THE HYPOPHYSECTOMIZED ALBINO RAT[1]

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SINCE Lacassagne (1) demonstrated a morphologic sex difference of submaxillary glands in mice, a large number of studies and experiments have accumulated which indicate a hormonal control of at least some of the functions of these glands in rats and mice.

It has been shown that hypophysectomy (2), thyroidectomy (3), castration (4), and a combination of castration and thyroidectomy (5) lead to a relative reduction of the intralobular ducts in the submaxillary gland of rats. Changes after thyroidectomy, castration, and castration-thyroidectomy were reversed following injections of thyroxine, testosterone, and the combination of the two hormones. It was further demonstrated that hypophysectomy results in the reduction of proteolytic activity of these glands (2).

The present study was undertaken to study the effect of testosterone and thyroxine on the enzymatic action of the submaxillary gland of hypophysectomized rats and to correlate these findings with the morphologic changes of the glands.

METHODS AND MATERIALS

Thirty-five male rats of the Sprague-Dawley strain were used. The animals were 120 days old at the time of hypophysectomy. Starting from the day after the operation, the experimental animals were injected twice daily for 30 days as follows:[2]

Group I—0.25 mg. of testosterone propionate in 0.1 cc. of saline solution containing 1% duponol C.

Group II—3 micrograms of dl-sodium thyroxine in 0.1 cc. of saline.

Group III—Both hormones in the same doses as Groups I and II.

Two control groups, one of intact animals, and the second of hypophysectomized animals given twice daily injections of 0.1 cc. of saline, were kept under the same conditions as the experimental animals. Each of these five groups consisted of seven animals. The animals were fed *ad libitum*.

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[1] This investigation was carried out under Contract DA-49-007-MD662 with the Medical Research and Development Board, Office of the Surgeon General of the United States Army.

[2] Dosage patterned after the work of Grad and Leblond (5).

After 30 days of treatment, the animals were killed with ether, and the submaxillary gland dissected free, placed in liquid nitrogen, then lyophilized in a freeze-drying unit. When it was dry, an extract was made by grinding the finely-cut frozen-dried gland in a mortar and slowly adding physiologic saline solution. Protease activity was determined using the method of Kunitz (6). Amylase activity was determined using the method of Myers, Free, and Rosinski (7). Saturating substrate conditions and optimal pH were determined for the intact group of control animals, and these conditions were employed for the assays in the other groups. These assay conditions were as follows:

	Protease	Amylase
Substrate	Casein	Starch
Substrate Concentration	1%	0.65%
pH	9.6	7.0
Incubation time, min.	30	15
Extract Concentration (dry weight)	5 mg./cc.	4 mg./cc.
Temp. (° C)	37	37

Nitrogen determinations were made according to the method of Ma and Zuazaga (8). One submaxillary gland from every animal was used for histologic study. This was fixed in 10% formalin and stained with hematoxylin and eosin.

FINDINGS

1. Body and Gland Weights

Average body weights at the time of hypophysectomy and body and submaxillary gland weights after 30 days of hormone administration are listed in Table 1.

TABLE 1. AVERAGE WEIGHT OF ANIMALS AND OF SUBMAXILLARY GLAND

Treatment	Initial body weight (gm.)	Final body weight (gm.)	Submaxillary gland (dry weight) (mg.)	Ratio of gland to body weight (×1000)
None (Normal)	350	400	76.9	.192
Hypophysectomy	350	255	28.2	.111
Testosterone	347	254	43.0	.169
Thyroxine	347	246	41.0	.169
Testosterone + Thyroxine	347	277	55.5	.200

All three of the hormone-treated groups showed the same drastic weight loss as the hypophysectomized animals. The submaxillary gland, however, decreased out of proportion to the body weight in the hypophysectomized group. It approached its normal ratio under both single hormone treatments and recovered it fully under the combined treatment.

2. Enzyme Activity

The average specific activities for the proteolytic and amylolytic enzymes are presented in Table 2. For the intact animals, proteolytic activity averaged 0.68 units of optic density per mg. N; hypophysectomy

reduced the value to one-third. The proteolytic activity was partially restored in the animals given either testosterone (activity = 0.36) or thyroxine (activity = 0.37). The combined treatment almost brought about a return to that in normal animals (average activity = 0.62). For amylase, hypophysectomy reduced activity from .36 to .15 units, testosterone had a marked depressing effect (average activity = 0.05), and thyroxine alone had no effect (average activity = 0.15). In combination, testosterone and thyroxine had a synergistic effect (average activity = 0.44), and restored the gland to normal activity.

TABLE 2. ENZYME ACTIVITIES OF THE SUBMAXILLARY GLAND EXTRACTS IN THE EXPERIMENTAL AND CONTROL GROUPS

Treatment	Protease		Amylase	
	Enzyme activity (O.D./mg N)	Standard deviation	Enzyme activity (O.D./mg N)	Standard deviation
None (Normal	0.68	.066	0.36	.199
Hypophysectomy	0.23	.047	0.15	.102
Testosterone	0.36	.088	0.05	.032
Thyroxine	0.37	.093	0.15	.025
Testosterone + Thyroxine	0.62	.045	0.44	.428

Probability values:

Protease (1) Hypophysectomized vs Testosterone: $P < .01$
(2) Hypophysectomized vs Thyroxine: $P < .01$

Amylase (1) Normal vs Hypophysectomized: $P < .05$
(2) Hypophysectomized vs Testosterone: $P < .05$
(3) Testosterone vs Thyroxine: $P < .05$
(4) Hypophysectomized vs Testosterone + Thyroxine: $P > .05$

3. *Microscopic Findings*

The parenchyma of the submaxillary gland of the rat consists of acini, intercalated ducts, intralobular ducts[3] containing secretory granules, and excretory ducts. The acini constitute approximately 60 to 70 volumes per cent of the gland. Most of the remaining 30 to 40% consist of intralobular duct tissue.

The morphologic changes following hypophysectomy were the same as those observed previously by Sreebny (2). There was a marked reduction in the diameter of the intralobular ducts and change of their high columnar cells into low cuboidal cells, which could no longer be distinguished from the lining of the smaller excretory ducts. The acini did not show any marked changes. The administration of testosterone alone and of thyroxine alone brought about a partial reversal of the changes in diameter and shape of the cells of the intralobular ducts, and the reappearance of some secretory granules. The simultaneous administration of both hormones restored

[3] The term "intralobular ducts" used herein is identical with "serous tubules" of C. P. Leblond *et al.*

the gland to near normal appearance and to the normal proportion of intralobular duct tissue.

A quantitative estimate confirmed the latter impression. In two specimens from each experimental group the area occupied by intralobular ducts containing secretory granules was estimated in a histological section. In 1080 squares examined, the number of squares occupied by such tissue varied as follows:

	First Specimen	*Second Specimen*
Normal	275	283
Hypophysectomized	None	None
Testosterone	110	110
Thyroxine	133	195
Testosterone + Thyroxine	244	248

DISCUSSION

Studies of castration in combination with thyroidectomy by Grad and Leblond (5) and of hypophysectomy have made it likely that these two experimental procedures lead to the same atrophic changes in the submaxillary gland of the rat. The changes are seen chiefly in the intralobular portion of the gland. They consist in disappearance of the secretory granules (all authors), in reduction in cell size and diameter of tubules (5), and in reduction of the proportion of parenchymatous tissue composed of intralobular duct cells (2), and the present study. Grad and Leblond have shown that the atrophy following thyroidectomy and castration can be reversed by the administration of thyroxine and testosterone. The present study has shown that the same two hormones can reverse the effect of hypophysectomy also, which makes it likely that the atrophic changes induced by hypophysectomy are due to the lack of the gonado- and thyrotrophic hormones.

Sreebny (2) and Shafer and Muhler (9) had further shown that a drop in proteolytic and amylolytic activity of the submaxillary gland is associated with the atrophic changes induced by hypophysectomy. As the present study shows, these changes also can be reversed by the combined action of thyroxine and testosterone. Thus, the pituitary control of both morphological and functional aspects of the submaxillary gland appears to be mediated by the testis and thyroid.

The submaxillary gland of the hypophysectomized animal has only half the relative weight as the submaxillary of the intact animal. Hormone treatment, singly or combined, did not influence the sharp loss in total body weight which follows hypophysectomy. Both single hormones, however, led to partial restoration of the relative weight of the submaxillary gland, and the combined treatment led to full restoration. It is interesting to note that the single hormones restored the weight ratio and the proteolytic activity to precisely the same level. No hormone treatment may be

considered as full replacement for the pituitary hormones as long as the body weight of the animal remains sharply reduced. But the differential effect of the pituitary hormones on morphology and enzyme activity of the submaxillary gland may be considered as fully replaceable by the combination of testosterone and thyroxine.

A difference in the hormonal control of proteolytic and amylolytic activity is indicated by the finding that the former is partially restored by testosterone or thyroxine, whereas the latter is depressed by testosterone and is unaffected by thyroxine. The nature of the synergistic action of testosterone and thyroxine in restoring amylolytic activity when combined remains unexplained.

SUMMARY AND CONCLUSIONS

Testosterone and thyroxine were administered for a thirty-day period to rats hypophysectomized at 120 days of age. Qualitative and quantitative aspects of the morphology and the enzyme activity of the submaxillary gland were studied.

The atrophy which follows hypophysectomy and the accompanying reduction in proteolytic and amylolytic activity of the gland could be reversed following the combined administration of testosterone and thyroxine.

A difference in the hormonal control of proteolytic and amylolytic activity is indicated by the finding that the former is partially restored by testosterone or thyroxine, whereas the latter is depressed by testosterone and is unaffected by thyroxine.

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THE EFFECT OF TEMPERATURE ON THE POTENTIATION OF ADRENALIN BY THYROXINE IN THE ALBINO RAT[1,2]

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IT WAS shown in a previous paper (1) that at 30° C, ambient temperature, thyroidectomy inhibits and injected thyroxine potentiates the calorigenic action of adrenalin, the potentiation being proportional to the daily thyroxine dose. In view of the great stimulation of metabolism by cold and the disputed role of the two hormones in this response, it seemed of interest to re-examine the interrelation between constant doses of the two hormones during short or long cold exposure.

The two- to three-fold rise in metabolic rate produced by sudden exposure of animals to near-freezing temperatures has been mainly attributed to muscular activity (2, 3), supplemented by adrenalin secretion (4, 5, 6, 7, 8). On the other hand, cold adaptation, characterized by maintenance of normal rectal temperature (2, 9, 10, 11) and greater resistance to more severe cold (12, 13, 14), is largely associated with increased thyroid activity (5, 15, 16).

Although it has repeatedly been demonstrated that thyroidectomized animals cannot survive in the cold (17, 18, 19, 20), whether the thyroid is directly responsible for the phenomenon of adaptation is still in doubt. The rise in basal metabolism (measured at 28° to 30° C) produced by the increase in thyroxine after prolonged cold exposure is relatively small when compared to the total increase in metabolism, which may be maintained at a double or triple rate and further increased by short exposure to more severe cold (11, 19, 21). Since thyroxine strikingly potentiates the calorigenic action of adrenalin (1), Ring's (5) suggestion that the main role of thyroxine in cold adaptation is the potentiation of adrenalin seems appropriate.

To assess the relative roles of thyroxine and adrenalin in cold adaptation, the influence of low temperature on the interrelation between them was studied. This experiment was designed to measure the changes in

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oxygen consumption produced by a standard adrenalin dose at various levels of thyroxine and at several different temperatures.

METHODS

One hundred and twenty male Sprague-Dawley rats, weighing 130 ± 10 gm., were divided into two series. Series "A" was kept at 26° C ± 2°, and series "B" at 5° C ± 1°. Each series consisted of five groups: intact controls and thyroidectomized groups receiving 0, 6 μg., 24 μg., and 48 μg. of l-thyroxine per animal[3,4] daily. The animals of series "B" were placed in the cold on the day following thyroidectomy. Because the untreated thyroidectomized rats could not survive longer than a week in the cold, no data for this group were obtained. In order to produce a maximum degree of thyroid deficiency, the ingestion of exogenous thyroxine and the synthesis of extra-thyroidal thyroxine were minimized by feeding the animals an iodine- and thyroxine-deficient diet (20) throughout the experimental period. Completeness of thyroidectomy was indicated by the fact that the animals ceased to grow 2 weeks postoperatively (22).

Oxygen consumption was measured during the third through the sixth week after thyroidectomy, by the method described by Grad (23). The metabolic rate of all rats was determined at 30° C ± 1° and at 10° C ± 1°, and additional measurements were taken on series "B" at 18° C ± 1°. The rats were placed in the glass jars used for metabolic rate determinations 1 hour before oxygen consumption was recorded. On alternate days, adrenalin injection immediately preceded insertion of the animals into the jars.

In preliminary experiments, in which oxygen consumption was measured at frequent intervals over a 4-hour period, the response to adrenalin[5] was maximal at 1 hour, and oxygen consumption then slowly returned to normal by 4 hours, regardless of the ambient temperature. Therefore, the metabolic rate 1 hour after intramuscular injection of a suitable dose of adrenalin was used as a standard measure of the calorigenic activity of adrenalin. The dose chosen was 60 μg./100 gm. body weight,[6] a dose intermediate between those (40 μg. and 100 μg.) used in our previous study of thyroxine-adrenalin interrelations in warm environments (1), and at the same order of magnitude as used by Ring (5) (50 μg./100 gm. of adrenalin-gelatin) and by Bloom and Russel (24) (20–200 μg./100 gm. of adrenalin bitartrate S.C.).

RESULTS

At each temperature, the oxygen consumptions of cold- and warm-adapted rats receiving similar doses of thyroxine did not differ significantly (Table 1). In preparation of the figures, advantage was taken of this fact by combining data from comparable groups excepting controls, in both series. The results from control groups of course differed since endogenous secretion of thyroxine was affected by previous exposure.

When measured at 30° C, the oxygen consumption varied directly as the log dose thyroxine (Fig. 1), and adrenalin increased the slope of the line relating oxygen consumption to thyroxine dosage. In animals virtually

[3] All doses of thyroxine will subsequently be expressed as μg./animal daily.

[4] Squibb-90%, l-thyroxine, in slightly alkaline saline solution.

[5] Parke, Davis, 1 cc. ampoules of adrenalin-in-oil, 1:500. Adrenalin-in-oil was found preferable to adrenalin chloride because its slower absorption prevented local necrosis and produced more uniform results.

[6] All doses of adrenalin will subsequently be expressed at μg./100 gm. body weight.

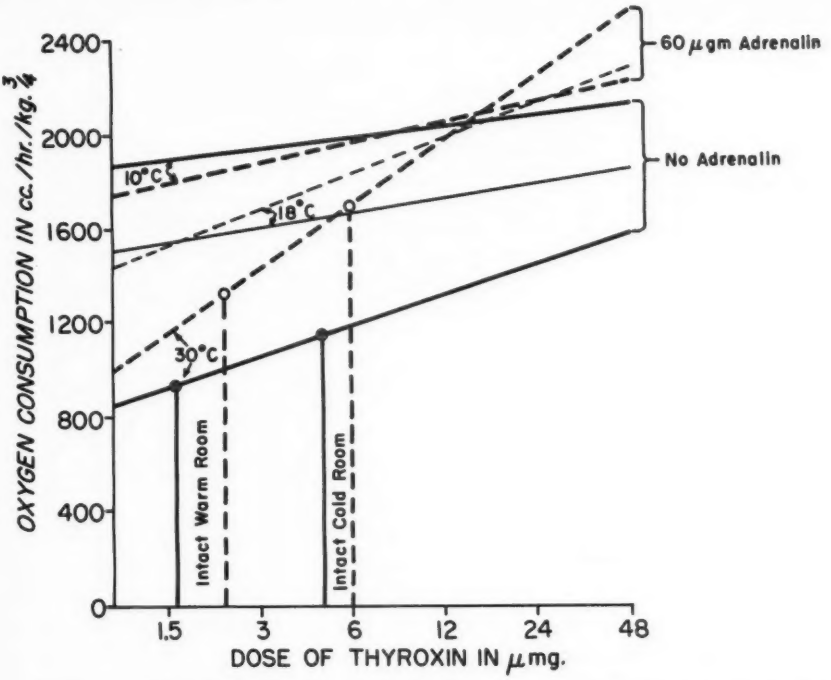

FIG. 1. Regression of oxygen consumption by thyroidectomized rats treated with various doses of thyroxine plotted against the dose of thyroxine on a logarithmic scale. For completeness, thyroidectomized animals were considered to secrete 1 μg. of thyroxine.[7] Measurements were taken at 30°, 18°, and 10° C. Solid lines represent oxygen consumption without adrenalin. Dotted lines represent oxygen consumption 1 hour after 60 μg. of adrenalin/100 gm. body weight, i.m. ⊗ and ○ represent oxygen consumption of intact controls, without and with adrenalin repsectively. Since (except in intact controls) there was no difference between cold- and warm-adapted animals, the data from corresponding groups were combined.

lacking thyroxine,[7] adrenalin had no significant calorigenic effect. These results confirmed those of a previous paper (1) where it was shown that the slope was directly related to the dose of adrenalin.

When measured at 10° C, oxygen consumption was significantly higher than at 30° C, and the slope was significantly reduced. Untreated thyroidectomized animals reached this high level of metabolism during their first exposure, but could not maintain it for longer than 90 minutes, nor even attain it upon repeated exposure. Increased metabolism in thyroidectomized animals may result only from muscular activity, while in thy-

[7] Leblond and Eartly (20) postulated a minimal amount of circulating thyroxine even in completely thyroidectomized rats on an iodine-deficient diet.

TABLE 1. OXYGEN CONSUMPTION OF INTACT AND THYROIDECTOMIZED RATS, RECEIVING THYROXINE AND ADRENALIN, MEASURED AT 30°, 18° AND 10° C.

	Group**	Daily Dose Thyroxine	Oxygen Consumption (cc./hr./kg.$^{\frac{3}{4}}$) 30° C.		18° C		10° C	
		μg.	μg. Adrenalin		μg. Adrenalin		μg. Adrenalin	
			0	60	0	60	0	60
Series A (Adapted to 26°C)	Intact	0	925 ± 25*	1361 ± 66			1900 ± 105	1827 ± 56
	Operated	0	774 ± 31	824 ± 37			1866 ± 57	1750 ± 63
	Operated	6	1146 ± 46	1997 ± 57			2033 ± 62	2046 ± 68
	Operated	24	1485 ± 41	2186 ± 75			2074 ± 80	2218 ± 53
	Operated	48	1553 ± 70	2421 ± 101			2227 ± 81	2227 ± 90
Series B (Adapted to 5° C)	Intact	0	1164 ± 66	1690 ± 66	1543 ± 51	1940 ± 58	1735 ± 48	1778 ± 49
	Operated	6	1215 ± 54	1835 ± 58	1795 ± 36	1827 ± 78	2036 ± 58	1967 ± 60
	Operated	24	1366 ± 27	2238 ± 53	1664 ± 77	2052 ± 65	1969 ± 68	2197 ± 48
	Operated	48	1645 ± 121		1908 ± 68	2415 ± 64	2066 ± 79	2139 ± 96

* Standard error.
** The number of animals per group was 12.
Statistical significance: (Analysis of Variance).
No significant difference between animals kept at 26° C and at 5° C except intact.
Individual treatments with thyroxine, adrenalin and temperature were very highly significant.
Interaction between any two of these treatments was very highly significant.
Interaction between all three treatments was significant.

roxine-treated rats, endogenous adrenalin may produce enough heat to replace muscular activity partially or completely.

The effect of exogenous adrenalin was much less striking at 10° C than at 30° C, and 60 μg. produced no significant increase. The time course was similar to that at 30° C, the maximal response occurring after approximately 1 hour. Additional experiments showed that a dose of 100 μg. of adrenalin was fatal at 30° C to all animals receiving more than 3 μg. of thyroxine. At 10° C this dose of adrenalin was well tolerated by rats receiving as much as 24 μg. of thyroxine. In the warm, adrenalin-treated animals appeared in a state of exhaustion; they showed tachypnea and also increased urination and defecation. If kept cool, the animals stayed in much better condition.

The relative ineffectiveness of moderate adrenalin administration at 10° C may possibly be attributed to substitution for the animal's endogenous adrenalin secretion, although there is no direct evidence for a diminution of secretion. Perhaps the greater tolerance to excessive adrenalin in the cold is related to the greater heat dissipation. At an external temperature of 30° C, the great heat production resulting from the synergistic action of adrenalin and thyroxine in large doses may produce hyperthermia. This increase in body temperature may in itself cause a further rise in metabolism which may account for the greater calorigenic action of 60 μg. of adrenalin at 30° C than at 10° C (cross-over of curves in Fig. 1), and result in death if the adrenalin dose is increased. Conversely, the decrease in metabolism produced by adrenalin in untreated thyroidectomized rats observed in previous studies (1), may be the result of hypothermia produced by muscular vasodilation.

The metabolic measurements carried out at 18° C showed an absolute level of oxygen consumption and slope intermediate between those obtained at 30° C and at 10° C (Fig. 1). The inverse relationship between

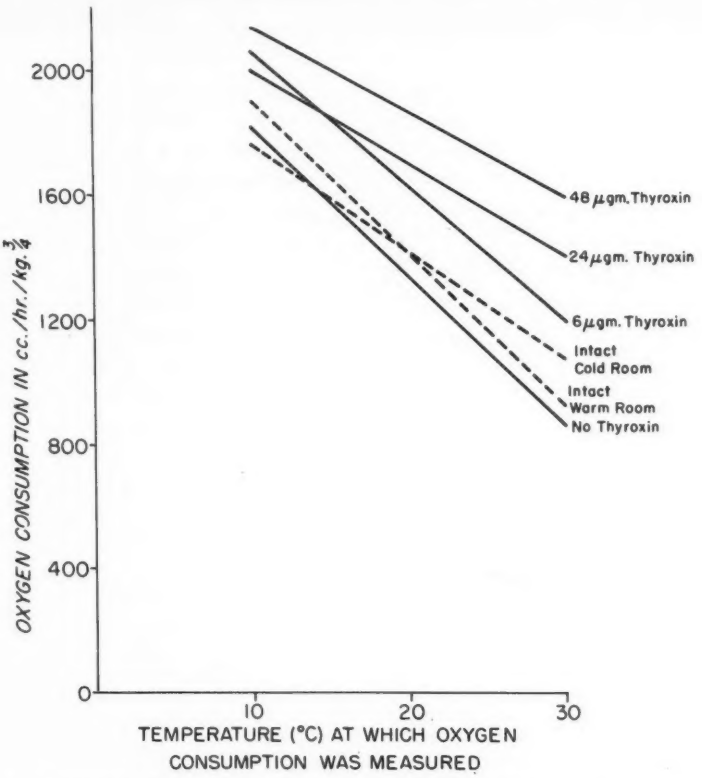

FIG. 2. Regression of oxygen consumption of thyroidectomized rats treated with various doses of thyroxine versus temperature at which oxygen consumption was measured. Solid lines represent thyroidectomized animals treated with indicated doses of thyroxine. Dotted lines represent intact controls. Data from cold- and warm-adapted animals (except intact controls) were combined.

oxygen consumption and temperature is illustrated in Figure 2. The differences between all thyroxine levels decreased with decreasing temperature. The effect of temperature on the response to adrenalin is even more striking (Fig. 3), clearly indicating that the calorigenic action of adrenalin can produce useful heat in the cold. The positive slope of the 48 μg.-thyroxine curve may be attributed to the previously suggested hyperthermia in the warm.

In intact rats, whose thyroxine level was not fixed, the oxygen consumptions of cold-adapted and non-adapted rats, measured at 30° C, differed significantly. When these measurements were interpolated on the thyroxine dose-response curve, thyroxine secretion appeared to have been increased from approximately 2.0 μg. to 5.5 μg./day. The response to exogenous adrenalin at 30° C was consistent with the presumed thyroxine

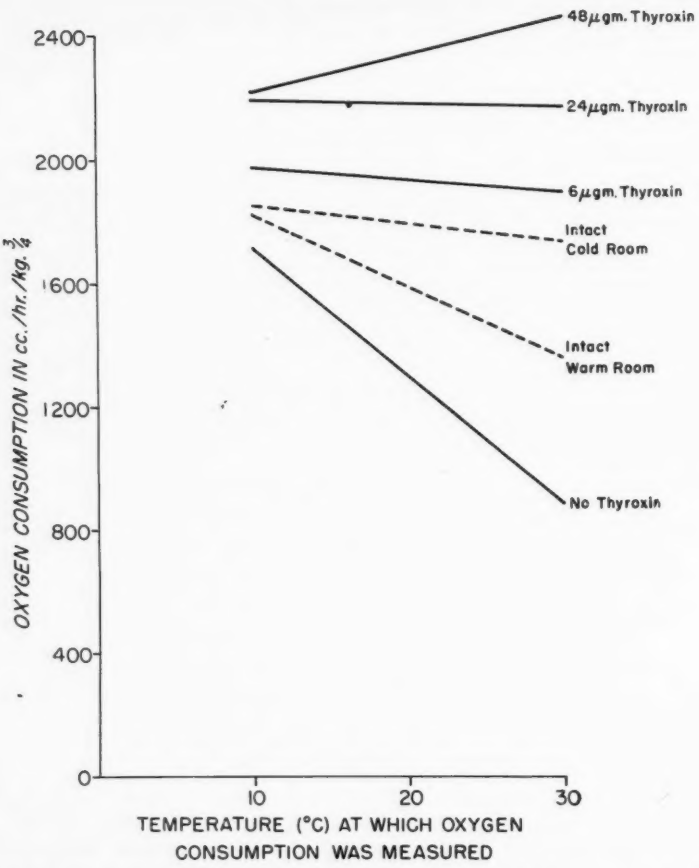

FIG. 3. Regression of oxygen consumption of thyroidectomized rats treated with various doses of thyroxine, 1 hour after 60 μg. of adrenalin/100 gm. body weight, i.m., versus the temperature at which oxygen consumption was measured. Solid lines represent thyroidectomized animals treated with indicated doses of thyroxine. Dotted lines represent intact controls. Data from cold- and warm-adapted animals (except intact controls) were combined.

levels. At 10° C, however, the oxygen consumptions of cold-adapted and non-adapted rats with or without adrenalin were not significantly different (Figs. 2, 3).

DISCUSSION

Intact rats respond to prolonged cold exposure by increasing thyroxine secretion, an adjustment reflected by a rise in oxygen consumption at 30° C (a temperature slightly higher than thermal neutrality (25)). The thyroxine requirements of approximately 2.0 μg. and 5.5 μg. of l-thyroxine at 26° and 5° C respectively, calculated in the present experiments, agree

well with the data of Dempsey and Astwood (26), based on thyroid weight after thiouracil administration, and of Woods and Carlson (27), based on thyroid weight after thiouracil and on oxygen consumption after thyroidectomy. While untreated thyroidectomized animals cannot acclimatize to cold, Sellers and You (19) showed that a very small amount of thyroxine insufficient to maintain normal metabolism at 30° C, permitted survival of athyroid rats at 1.5° C. In spite of the fact that these animals received a constant small dose of thyroxine (2.5 μg. daily—presumably d,l) when they were exposed to cold the 30° C metabolism rose gradually as it did in normal animals. Although the level attained was not as high as in normal animals, the percentage increase was actually greater. Presumably thyroxine played a permissive role for the operation of some other factor (e.g., muscle tone). In the present experiment all dosages of thyroxine were above that required for normal function and at all temperatures the oxygen consumptions of treated rats did not differ significantly according to previous adaptation to a warm or cold environment.

The immediate response to cold exposure has been attributed to a combination of muscular activity and adrenalin secretion (2, 5, 6, 7, 8). The absolute calorigenic response to exogenous adrenalin in the present experiments was directly proportional to the log dose thyroxine, and cold-acclimatized intact rats showed an increased reactivity to adrenalin closely corresponding to their increased thyroxine secretion. It may be noted that the relative increase in intact or treated animals was nearly constant at any given temperature (150% at 30°; 125% at 18°; and 0% at 10°) and was the same for cold-adapted as for warm-adapted animals. These experiments, as well as work by Morin (6, 7) on the dog, also showed that cold exposure decreases the absolute response to exogenous adrenalin. This may indicate that the calorigenic action of adrenalin can be used to produce heat in the cold. Morin also demonstrated that in the absence of central nervous control (after spinal section), body temperature rises after adrenalin administration. He further observed that cold exposure induced a release of endogenous adrenalin as manifested in hyperglycemia and in acceleration of the denervated dog heart. Both responses were abolished by demedullation. Ring (5) observed that when exposed to 2.4° C, demedullated rats could reach the same level of metabolism as the controls, but could not maintain it. He attributed this to muscular fatigue and described the calorigenic role of adrenalin as partially substituting for muscular activity. Under more severe cold conditions adrenalin may supplement maximal action and allow the attainment of a higher absolute metabolic rate. Schaeffer (8) showed that intact rats could maintain their body temperatures at −7° C by increasing their calorigenic production 40% more than was possible for adrenalectomized rats treated with adrenal cortical extracts, which could not adjust to an ambient temperature of less than 6° C. Since the adrenal cortical extracts available at that time are

not as effective as cortisone, it is possible that these animals suffered some degree of adrenal cortical insufficiency.

If it is presumed that sudden exposure to cold elicits a maximal release of adrenalin, as suggested by Morin's work (6, 7) and by the refractoriness to exogenous adrenalin, then animals which are more reactive to adrenalin will require less muscular activity to reach the metabolic level needed for maintenance of homeothermy. Since reactivity to adrenalin is directly and intimately dependent on the thyroxine level, the main role of the increased thyroxine secretion in cold acclimatization may be, as Ring (5) suggested, the potentiation of adrenalin. The work of Sellers *et al.* (2) who found considerably less electrical activity in the skeletal muscle of cold-acclimatized rats than in that of non-acclimatized animals when both groups were exposed to 20°, 4° and 1.5° C offers some support for this hypothesis. The acclimatized group were also better able to maintain body temperature. Schaeffer (8) showed that the removal of both the thyroid and the adrenal medulla resulted in less cold resistance than the removal of either gland alone.

Evidence of direct mediation of thyroxine effects through adrenalin is presented by Brewster *et al.* (28). Feeding thyroid to thyroidectomized dogs for 3 weeks resulted in a rise in oxygen consumption, cardiac index and heart rate. Total sympathetic block (eliminating endogenous adrenalin) reduced all these indices to the level seen in blocked normal dogs. Adrenalin administered to these blocked dogs increased these indices in proportion to their thyroxine state.

The possible nature of the synergism between the two hormones has been extensively discussed in our previous paper (1).

SUMMARY

Oxygen consumption at 30°, 18° and 10° C was measured in several groups of thyroidectomized rats, maintained on fixed levels of thyroxine. The metabolic rate was found to vary inversely as the temperature.

At 30° C, the oxygen consumption was directly proportional to the log dose thyroxine and adrenalin increased the slope of the line relating oxygen consumption to thyroxine dosage.

At 10° C, the oxygen consumption at all thyroxine levels was much greater than at 30° C, and the slope decreased. Thyroidectomized rats could reach almost the same initial level as controls, but could not maintain it. The effect of adrenalin was much less pronounced at this temperature.

At 18° C, the results were intermediate between those obtained at 30° C and at 10° C.

Intact animals responded to 2 to 5 weeks cold exposure by an estimated increase in thyroxine secretion from 2.0 μg. to 5.5 μg. daily. This

was reflected in a higher oxygen consumption at 30°C. The oxygen consumption after adrenalin was also correspondingly higher.

It is postulated that the main role of thyroxine in cold acclimatization is the potentiation of the calorigenic action of endogenous adrenalin.

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THE BIOSYNTHESIS OF ALLOPREGNANE-3β,16α,20β-TRIOL FROM ACETATE-1-C^{14} IN THE PREGNANT MARE

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THE incorporation of acetate-1-C^{14} into estrone, equilin, and equilenin in the pregnant mare has been reported previously (1). The relatively high C^{14} content of the non-volatile phenolic non-ketonic fraction of the organic portion of the urine of this acetate-treated mare prompted a search for low molecular weight phenols which might function as intermediates in the anabolism or catabolism of the estrogens. In the course of this attempt, a neutral steroid was encountered and found to be highly radioactive. This proved to be the original "pregnanetriol" of Smith, Hughes, Marrian, and Haslewood (2), the constitution of which was later established by Hirschmann, Hirschmann, and Daus (3) as allopregnane-3β, 16α,20β-triol. Its isolation, identification, and significance are dealt with in the present communication.

EXPERIMENTAL AND RESULTS

Melting points were taken on the Fisher-Johns microblock; the recorded values are corrected. Radioactivity determinations were done as described previously (1).

The administration of radio-acetate to the pregnant mare and the initial fractionation of the urine have been previously reported (1). The following experimental procedure is in continuation.

Fractionation of non-ketones of non-volatile strong phenols (day 4)

The non-ketonic portion of the non-volatile strong phenolic fraction (day 4) was dissolved as completely as possible in 360 ml. of warm benzene. The benzene-soluble material was then partitioned into acidic, phenolic, and neutral fractions by extraction (3$\times\frac{1}{2}$ volume) with 5% sodium carbonate and 1N sodium hydroxide solutions respectively. The acidified carbonate and alkali solutions were then extracted (3$\times\frac{1}{2}$ volume) with ether and the combined ether extracts of each fraction and the remaining benzene were washed with water (each, 3$\times$0.1 volume) and distilled to dryness. Quantitative data and radio-assays are given in Table 1.

Isolation of allopregnane-3β,16α,20β-triol

The semicrystalline neutral material (362.5 mg.; specific activity,[1] 352) was triturated twice with 2 and 20 ml. of acetone and the crystals (19.2

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[1] Specific activity is defined as counts per minute per milligram.

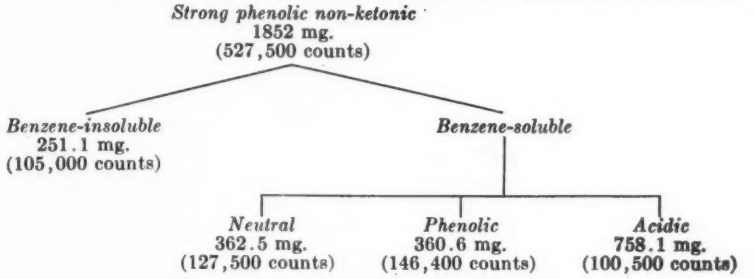
mg.) remaining had m.p. 300° C (decomposition) and specific activity, 1232. Identification was provided by a comparison (Fig. 1) of the absorption spectrum in sulfuric acid of the isolated crystals with that of authentic allopregnane-3β,16α,20β-triol.[2]

Attempts to isolate the compound from the non-ketonic portion of the non-volatile strong phenolic fraction (days 1 to 10 except day 4) were unsuccessful.

Preparation of allopregnane-3β,16α,20β-triol triacetate

A portion (14.1 mg.) of the allopregnane-3β,16α,20β-triolwas acetylated in a mixture of pyridine-acetic anhydride, as described by Hasle-

TABLE 1. FRACTIONATION OF NON-KETONES OF NON-VOLATILE STRONG PHENOLS (DAY 4)



wood, Marrian and Smith (4) to give 8.9 mg. of triacetate (m.p. and admixture m.p., 168°–169° C; specific activity, 885; calculated as the free triol, 1276). Further identification is provided by the infrared transmission curves (Fig. 2).

DISCUSSION

The results make it evident that acetate-1-C^{14} is readily incorporated into allopregnane-3β,16α, 20β-triol in the pregnant mare; radiochemical purity was established by conversion of the free triol to the triacetate, during which operation no diminution in specific activity was observed. The isolation of this neutral steroid from the "strong phenolic" fraction must be regarded as fortuitous, possibly the result of "carry over" of this extremely insoluble substance in light phenolic oils. Re-partition of the crude phenolic extract between solutions of sodium carbonate, sodium hydroxide and benzene yielded the steroid alcohol in the neutral fraction.

It is noted that the specific activities of allopregnane-3β,16α,20β-triol and estrone from the same day's output, 1,232 and 1,117 (1) respectively, are approximately the same.

[2] The authentic triol employed was isolated by the senior author from several hundred gallons of mares' pregnancy urine in 1936; at that time it had been compared directly and found identical with a specimen of the original "pregnanetriol" kindly provided by Prof. G. F. Marrian.

Seven 16α-hydroxylated steroids have now been isolated, all from human urine with the exception of the present mares' urine triol. The others are: estriol, Δ^5-androstene-3β,16α,17β-triol(5), Δ^5-pregnene-3β,16α,20α-triol (6), and etiocholane-3β,16α,17β-triol, androstane-3β,16α,17β-triol, and

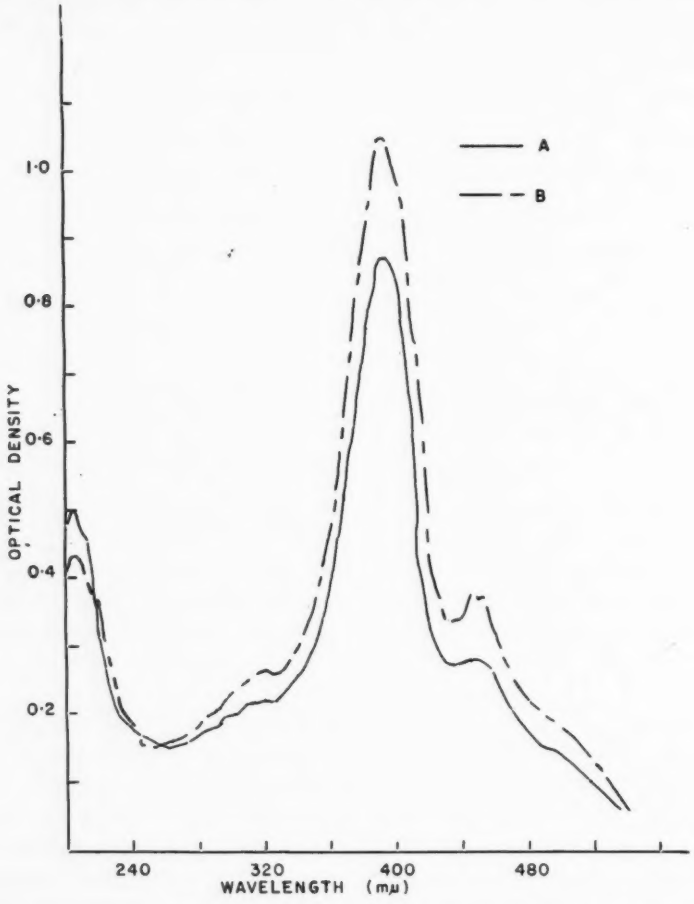


FIG. 1. Absorption spectrum in sulfuric acid of allopregnane-3β,16α,20β-triol. Curve A, isolated product. Curve B, authentic sample.

allopregnane-3β,16α,20α-triol (7). Apart from estriol, their metabolic significance is obscure. It has been suggested by the Hirschmanns (6) that the unsaturated triols originate from adrenal tumor tissue (or metastases), as they were encountered in the urine of such subjects but not in normal urine.

Lieberman and Teich (8) regard the saturated C_{19}-triols as "further steps

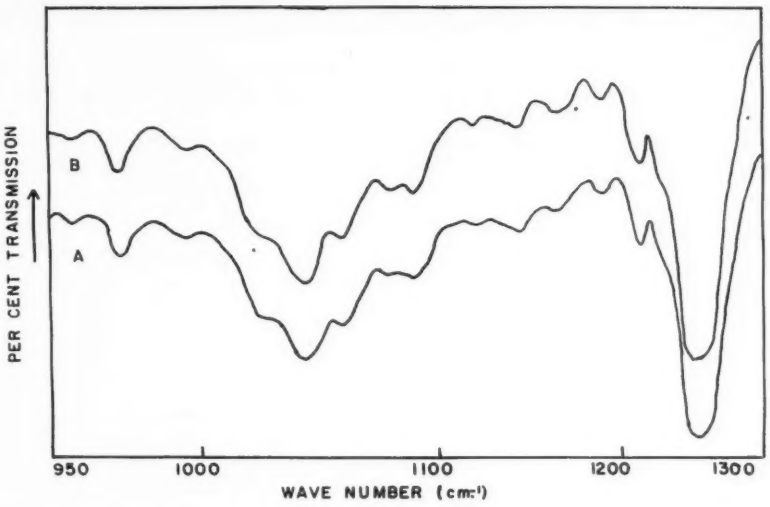


FIG. 2. Infrared transmission spectra in CS_2 (1 mm. cell) of authentic allopregnane-3β,16α,20β-triol triacetate (Curve A; 11.9 mg. in 2.385 gm. of solvent) and the acetylated isolated product (Curve B; 8.9 mg. in 1.825 gm. of solvent).

in the catabolism of steroids, preliminary to formation of more highly degraded products." The regular occurrence of allopregnane 3β,16α,20β-triol in mares' pregnancy urine in relatively large quantities would however suggest that, in this species at least, 16-hydroxylated steroids may well be elaborated by the normal adrenal cortex.

SUMMARY

Allopregnane-3β,16α,20β-triol was isolated from the urine (day 4) of a pregnant mare treated with acetate-1-C^{14} and found to be radioactive. The specific activity of this metabolite and that of estrone put out on the same day are approximately equal.

Acknowledgments

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CHLORIDE BALANCE IN HYPOPHYSECTOMIZED FROGS

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HYPOPHYSECTOMY in frogs and toads results in decreasing concentrations of sodium and chloride ions in muscles and body fluids (1, 2, 3, 4). The fall in plasma chloride in the frog *Rana temporaria* has been ascribed to increased permeability of the skin to chloride ions (4). However, we later found that the total chloride content diminished in hypophysectomized frogs even when the permeability of the skin to chloride ions remained unchanged. The chloride balance after hypophysectomy has therefore been studied in more detail in order to find out which factors are responsible for the decrease in body chloride.

MATERIAL AND TECHNIQUE

Four healthy specimens of the frog *Rana esculenta* (L.) were used for hypophysectomy. In one frog, operated upon in September, only the pars distalis of the hypophysis was extirpated. The other three frogs, one operated upon in September and two in December, were totally hypophysectomized. The operations were done under urethane anesthesia. The base of the brain was exposed by removing the parasphenoid, and the hypophysis was removed by suction. The operation was completed by replacing the parasphenoid and closing the wound with two stitches. The frogs were kept singly in wire cages in glass beakers with 100 cc. tap water, which was regularly changed. They were kept starving in an unheated basement room at temperatures about 15–17° C.

Changes in the chloride content of the frogs were followed by measuring changes in the chloride content of the water in which the animals were living. Chloride was determined on 1 cc. samples by Schnohr's modification of Rehberg's method (5). Total exchangeable body chloride was determined by means of the isotope dilution method. Cl^{36}-labelled sodium chloride was added to the water surrounding the frogs and changes in specific activity of the chloride in the water were followed. From the degree of dilution of Cl^{36} the amount of chloride in water plus frog was computed. Radioactivity was measured in evaporated 0.5 cc. samples by means of a Geiger-Müller end-window counter. Influx of chloride ions was calculated according to the equation derived by Koefoed-Johnsen and Ussing (6).

$$M = 2.3\frac{V_1 \cdot V_2}{t \cdot (V_1 + V_2)} \log \frac{aV_1 - bV_2}{aV_1 - bV_2 - Q(V_1 + V_2)}.$$

V_1 and V_2 are the amounts of chloride in the medium and inside the frogs respectively; a and b are the amounts of radioactive chloride present in the medium and the frog respectively, at zero time; Q is the amount of radioactive chloride which has entered through the skin of the frog in the time t. It was found that the small net changes occurring in chloride content of the medium and the frog did not invalidate the use of the equation which is valid only if the amounts of chloride in the two compartments remain constant. The mean values of chloride in medium and frog during the period

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considered were used in the calculations. Rates of water uptake and urine flow were determined by weighing as described previously (7).

It is known that neurohypophysial extracts enhance the uptake of sodium and chloride through the isolated or intact skin of various amphibians (8, 9, 10, 11). The reaction of the chloride deficient hypophysectomized frogs to chloride was therefore investigated in animals that were stimulated to increased chloride uptake by subcutaneous implantation of neurohypophysial tissue from one or more frogs. Such implantation secures a protracted effect in contrast to an injection of extracts because the implanted tissue slowly liberates its active principles.

RESULTS

Effect of hypophysectomy on chloride balance. In the two frogs hypophysectomized in September total exchangeable body chloride two months later was 20.5 and 22.4 μ eq./gm. body weight. The two controls contained 29.5 and 31.3 μ eq./gm. The two frogs totally hypophysectomized in December lost about 20% of their chloride content during the three weeks following the operation. The loss was not constant throughout these three weeks, and periods of net uptake of chloride occurred. There was no increase in weight after the hypophysectomies. (In *Rana temporaria* the body weight was found to increase rapidly by about 15% after hypophysectomy (2).) Thus content and concentration of chloride fell in *Rana esculenta* after extirpation of the pars distalis or the whole hypophysis.

Rates of influx and net uptake of chloride ions were measured concurrently with the determinations of total body chloride. It appears from Table I that over a period of 7 days there was no measurable difference

TABLE 1. INFLUX AND NET UPTAKE OF CHLORIDE ION THROUGH THE SKIN OF UNDISTURBED HYPOPHYSECTOMIZED AND NORMAL FROGS MEASURED WITH Cl^{36}

	Weight in gm.	Cl^--content per gm. body weight	Influx and net uptake of Cl^- during a period of 7 days in μ eq./h./100 gm.								Total influx and net uptake μ eq./100 gm.	
			1 day		2 days		3–4 days		5–7 days			
			influx	*net*	*influx*	*net*	*influx*	*net*	*influx*	*net*	*influx*	*net*
Total hypophysectomy	48	22.4	3.36	−2.70	2.96	−1.56	1.39	−2.65	3.54	−1.83	432	−165
Pars distalis extirpated	39	20.5	0	−3.00	0	−1.05	4.23	+0.41	7.05	+2.36	715	+42
Normals	66	29.5	5.14	−0.42	0.98	−2.16	4.63	−0.59	5.02	+1.44	713	+9
	36	31.3	0.39	−5.02	11.80	+3.78	6.03	−0.70	5.15	+0.23	964	−5

between operated and normal frogs. In both groups, periods of net uptake as well as net loss of chloride occurred. Chloride influxes varied greatly. Influx minus net uptake of chloride which gives the outflux through the skin plus excretion of chloride through the kidneys, was not larger in the hypophysectomized than in the normal frogs.

Chloride and water balance in chloride-loaded hypophysectomized frogs. Stimulation of chloride uptake by implantation of neurohypophysial tissue often increased total body chloride by 10%. The maximum was a 25% increase in 3–5 days. The chloride gained was, however, always

rapidly lost, despite the antecedent depletion of chloride. At the time of the experiments two of the frogs had lost more than 30% of their body chloride, the two other frogs had a chloride deficit of about 20%.

In four experiments we followed not only changes in chloride content, but also body weight, water uptake, diuresis and chloride concentration in urine. The net uptake or loss of chloride through the skin was computed from the net change in total body chloride and simultaneous renal loss of chloride. The results of two typical experiments may be seen from Figure 1. Immediately after implantation of the neurohypophysis the weight increased due to faster water absorption through the skin and to oliguria. Water uptake, diuresis and body weight were restored to about normal values within 1–2 days. The first day after implantation chloride balance was not markedly altered. However, the second day the net uptake of chloride ions through the skin was strongly increased in all experiments. High rates of chloride uptake persisted for several days. The enhanced cutaneous chloride transport resulted in a gain of body chloride. The gain in body chloride eventually became smaller due to increased urinary excretion of chloride, manifested mainly as an increase in concentration. After 3 to 5 days the renal loss of chloride exceeded the cutaneous uptake and all the chloride gained was excreted. At this time the chloride concentration of the urine had again fallen to the previous low level.

The initial chloride retention was accompanied by water retention, and the subsequent loss of body chloride by increased water excretion. In order to see how much water accompanied gains and losses of body chloride the regression equations were calculated for changes in weight and corresponding changes in chloride content of the frogs. Values used for these calculations began with the second day after implantation of the neurohypophysis when it could be assumed that the immediate effect of the implantation on the water balance had vanished. In the two frogs with the total hypophysis or pars distalis extirpated about 4 months before the experiments the regression equations were found to be $y=0.0152x-0.110$ and $y=0.0116x-0.127$ ($y=$weight in grams and $x=$Cl in micro equivalents). This means that in these two frogs an average gain or loss of 66 and 86 μ eq. Cl corresponded to a change in body water of 1 gm. In the two frogs which had been hypophysectomized for only three weeks the equations were $y=0.00873x-0.018$ and $y=0.00686x-0.094$, and the ratios therefore 115 and 146 μ eq. Cl to 1 gm. of water. The equations further indicate a weight loss independent of chloride balance of 0.018 to 0.127 g. per 1–2 days, probably due to metabolic break-down of body tissues.

DISCUSSION

It was previously found (4) that in *Rana temporaria* the permeability to chloride ions of the isolated skin five months after hypophysectomy

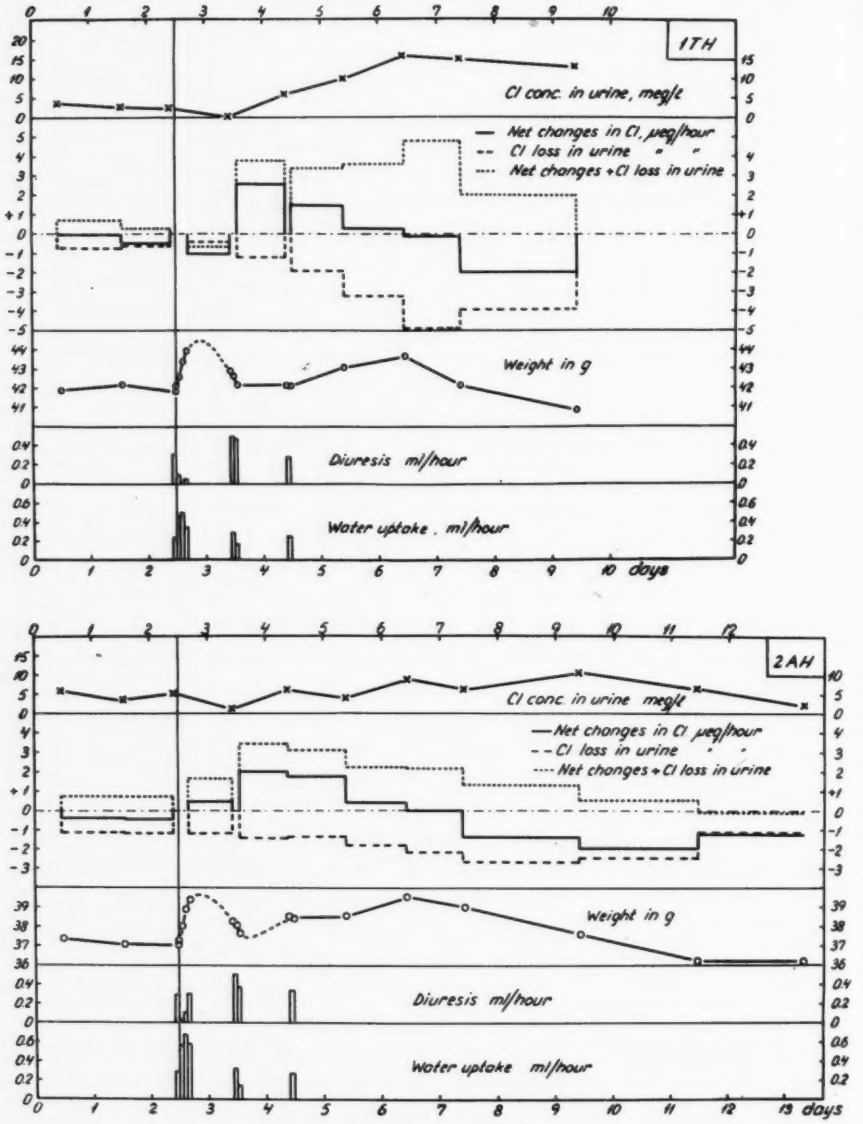

FIG. 1. Effect of subcutaneous implantation of frog neurohypophysis on balance of water and chloride ions in frogs four months after total hypophysectomy (1TH) or extirpation of pars distalis only (2AH). Vertical lines through the diagrams indicate time of implantations. The experiments were made at 15° C.

was much greater than that of the controls, whereas the ability to transport chloride ions was not influenced by the operation. It was therefore suggested that body chloride is lost in hypophysectomized frogs because the cutaneous chloride uptake can not keep pace with the loss through the skin and kidney. Increased permeability of the skin after hypophysectomy was further demonstrated in *Rana temporaria* (2) and *Rana pipiens* (12). However, in the present work it was found that the permeability to chloride ions of the skin of undisturbed *Rana esculenta* was not measurably changed two months after hypophysectomy even when at this time about one third of the total body chloride had been lost. Furthermore, after hypophysectomy chloride was not lost at a constant rate and periods of net uptake were encountered. Although, because of the great variability in the chloride fluxes, some increase in skin permeability can not be excluded, it is clear that there are other factors which cause much greater changes in chloride uptake and loss. Therefore altered permeability of the skin to chloride ion can not, alone, explain the fall in body chloride after hypophysectomy.

It is not clear why the skin permeability is not consistently found to be increased after hypophysectomy. However, it may be that the skin permeability of hypophysectomized frogs is increased only during moulting as in normal anurans (13, 7), and it is known that hypophysectomy increases the rate of cornification and moulting in anurans although to a widely varying extent in various species.[1]

It is noteworthy that the influx of chloride ion was about the same in the salt deficient hypophysectomized frogs as in the controls. In normal frogs salt depletion strongly increases the uptake of chloride ions through the skin (14, 15, 16). Either, therefore, the hypophysectomized frogs are unable to increase the chloride uptake according to their need, or a reduced chloride level does not act as stimulus to increased uptakes as in normal frogs. The last possibility appears the more likely, because salt deficient hypophysectomized frogs that were induced to take up chloride, reacted as if they were in normal chloride balance. Chloride uptake was accompanied by retention of water. Furthermore, the concentration of chloride in the urine rose, and all the chloride gained was finally excreted again together with the water retained. Thus the chloride taken up was treated as an excess of chloride despite the fact that the induced gain in chloride represented only a fraction of the existing deficit.

The two frogs with the largest losses of body chloride, and thus, presumably, also with the lowest chloride concentration in the plasma, showed the smallest changes in chloride content relative to changes in water content. This indicates that they adjusted their chloride concentration at a

[1] Terrestrial forms seem to be more strongly influenced than aquatic species. The effect of moulting on the permeability of the skin probably varies from species to species though exact investigations are lacking.

lower level than did the frogs with the smallest losses of chloride. It thus seems that hypophysectomized frogs regulate the chloride concentration of the extracellular fluid at progressively decreasing levels.

SUMMARY

In hypophysectomized frogs (*Rana esculenta* (L.)) there was a progressive reduction of total body chloride that was neither due to increased permeability of the skin to chloride nor to inability of the kidney to retain chloride. Chloride depletion in the hypophysectomized frogs did not induce increased influx of chloride ion through the skin as it does in normal frogs. When increased uptake of chloride ion was induced by implantation of neurohypophysial tissue, the hypophysectomized frogs reacted to the resulting gain in chloride content as to an excess of chloride, even when the gain was smaller than the existing deficit. It is suggested that the hypophysectomized frogs regulate the chloride ion concentration of the extracellular fluid at levels which decrease progressively after hypophysectomy.

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AN IMPROVED ASSAY METHOD FOR THYROTROPHIN USING DEPLETION OF I^{131} FROM THE THYROID OF DAY-OLD CHICKS

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WAHLBERG (1) has summarized most of the literature on assay of thyrotrophin (TSH) in various animals. Recently, Piotrowski, Steelman and Koch (2) described an assay method for TSH based on depletion of the stable iodine from chick thyroids, and Gilliland and Strudwick (3) proposed an assay method based on depletion of I^{131} from the thyroids of baby chicks which were also treated with thyroxine to minimize endogenous secretion of TSH. Albert *et al.* (4) have shown that propylthiouracil (PTU) increased the thyroid weight response to TSH in baby chicks. More recently Halmi *et al.* (5) (6), and VanderLaan and Caplan (7) have studied the effect of PTU on thyroid function in hypophysectomized rats and found PTU to accentuate the effect of TSH.

It has been found that both the accuracy and sensitivity of the I^{131} depletion method of assay in chicks are greatly increased when PTU and thyroxine are given simultaneously with the TSH. The method described herein has an index of precision (λ) of 0.20, which makes it possible to estimate potency with a standard error of $\pm 20\%$ using 26 chicks equally distributed among standard and unknown.

MATERIALS AND METHODS

Carrier free I^{131} was diluted with distilled water to the desired concentration (10–15 μc./ml.).

Thyroxine solution was prepared at a concentration of 1 mg./ml. and at pH 9.5–10. This was diluted further so that 0.2 ml. contained 8 μg. of thyroxine.

Solutions of PTU: 750 mg. or 75 mg. of propylthiouracil (PTU) was suspended in about 20 ml. of water with the aid of a drop of wetting agent. Just sufficient NaOH was added to dissolve the PTU and the total volume made to 30 ml. with water. Two-tenths ml. of this solution contains 5 mg. or 0.5 mg. of PTU. Originally, 5 mg. PTU/day was used and found to be somewhat toxic. Later, 0.5 mg. PTU/day was found to be just as effective with little toxicity.

Solution of PTU and thyroxine: Sufficient thyroxine solution (1.2 ml. = 1.2 mg.) was added to the PTU solution before making up to a volume of 30 ml. Two-tenths ml. of this auxiliary solution (A) contains 8 μg. of thyroxine and either 5 mg. or 0.5 mg. PTU. Solutions containing PTU were freshly prepared each week.

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Unless indicated, the TSH standard used was a preparation of growth hormone, C_5I_5, which has a potency of 0.2 USP units of TSH/mg.

Radioactivity in the thyroids of the chicks was measured *in vivo* by holding the chicks upside down with their thyroids over the 2½ cm. hole in the lead nose-piece collimator of a model DS-1[1] scintillation counter. Counts were of 1 minute duration. With this arrangement 1 μc. gives a count of about 2500/minute. By removing the lead collimator and counting on the end plate of the counter as little as 0.3–0.5 μc./chick can be used, since the count per μc. is increased by a factor of about 8.

New Hampshire red chicks were obtained the day after hatching.[2] From week to week these chicks have an average I^{131} uptake of 20 to 60% depending upon the time of year. For most experiments chicks were kept in a commercial brooder with water to drink, but with no food. The chicks which were fed received a complete synthetic diet (8), from which only the potassium iodide was omitted (C_7-KI). It contained about 0.1–0.2 mg. I/kg.

Procedure: As soon as possible after arrival, the chicks were divided into groups, usually 5 per group, so that the total body weight of each group was nearly the same. All chicks were then injected subcutaneously on the inner aspect of the thigh with 0.2 ml. (2 to 3 μc.) of the I^{131} solution. Twenty-four hours later, counts were made of the I^{131} activity in the thyroids. We have confirmed the report of Wahlberg (1) that uptake is approximately linear for 8 hours and rarely increases after 16 hours. The TSH solution to be assayed was then injected subcutaneously in a volume of 0.2 ml. on one side of the body and 0.2 ml. of the auxiliary solution A, containing PTU and thyroxine, was injected on the opposite side. This procedure of counting and of injecting both TSH and solution A was repeated daily for two or three days. On the final day, only a count was made. Routinely, the chicks were fasted. This eliminated possible variability due to food and iodine intake.

Calculation and statistical procedures

The results of a typical experiment on the effects of TSH injection on iodine depletion are shown in Table 1. In the first 4 columns we show the per cent of injected I^{131} remaining in the thyroid after 0, 1, 2, and 3 injections of TSH spaced at 24 hour intervals. Results are shown separately for individual chicks and at six different dose levels. All values are corrected for physical decay of the isotope. It will be noted that chicks with a high initial percentage tend to remain high after the administration of daily doses of TSH and those with low percentage tend to stay low.

In the last four columns of Table 1 we have expressed the I^{131} content as a per cent of the initial value, which was obtained 24 hours after injection of I^{131}, but before injection of anything else. In what follows we refer to these latter per cent values as relative per cent remaining (RPR) and for contrast refer to the per cent values in the first four columns as absolute per cent remaining (APR). Analysis of variance confirms what is obvious by inspection, and that is that the RPRs are much less variable than are the APRs, particularly at high values of RPR. By using RPR as the

[1] Manufactured by Nuclear Instrument and Chemical Corporation, Chicago 10, Illinois.

[2] Laurelvue Poultry Farm and Hatchery, Laurel, Maryland.

TABLE 1. ABSOLUTE AND RELATIVE PER CENT OF INJECTED I^{131} REMAINING IN THYROIDS OF INDIVIDUAL CHICKS BEFORE AND AFTER 1, 2, AND 3 DAYS INJECTION WITH STATED DOSE OF TSH. AFTER COUNT ON 0 DAY ALL CHICKS WERE INJECTED DAILY WITH SOLUTION A. ALL CHICKS WERE FASTED

Dose of TSH mu/day	No. of days injected with TSH							
	0	1	2	3	0	1	2	3
	Absolute per cent remaining of I^{131} (APR)				Relative per cent remaining of I^{131} (RPR)			
0	31.8	30.5	30.9	29.3	100	96	97	92
	55.3	45.5	50.0	43.8	100	82	90	79
	60.0	54.0	51.0	49.0	100	90	85	82
1.67	27.2	22.3	23.2	22.8	100	82	85	84
	40.0	38.0	34.5	31.2	100	95	86	78
	44.7	41.1	37.0	dead	100	92	83	dead
	27.8	24.4	23.6	dead	100	88	85	dead
	52.9	48.1	47.8	43.2	100	91	90	82
	62.7	61.6	54.5	48.8	100	98	87	78
	25.7	23.1	24.1	20.3	100	90	94	79
3.3	31.0	25.6	22.4	18.8	100	83	72	61
	44.5	40.1	34.2	32.1	100	90	77	72
	37.7	25.0	19.6	12.9	100	66	52	34
	33.2	22.8	18.7	16.8	100	69	56	51
	42.6	36.0	32.9	28.6	100	85	77	67
	36.0	26.0	25.0	20.8	100	72	69	58
6.7	43.6	30.9	24.2	14.0	100	71	56	32
	38.0	28.0	16.0	7.5	100	74	42	20
	35.0	29.1	27.4	21.7	100	83	78	62
	53.0	41.4	36.4	34.3	100	78	69	65
13.3	61.2	41.1	30.3	19.9	100	67	50	33
	27.8	12.3	7.8	5.3	100	44	28	19
	43.0	18.0	12.1	3.4	100	42	28	8
	35.9	13.7	5.3	4.4	100	38	15	12
	23.9	17.3	14.4	dead	100	72	60	dead
	44.5	21.4	15.2	9.3	100	48	34	21
	37.4	22.1	8.2	4.6	100	59	22	12
26.7	36.0	9.6	2.9		100	27	8	
	53.3	17.5	10.0	not	100	33	19	not
	41.8	9.5	4.7	in-	100	23	11	in-
	42.8	15.6	4.3	jected	100	36	10	jected
	14.3	8.2	5.0		100	57	35	
	38.6	14.5	5.9		100	38	15	

measure of the biological response, each chick serves as its own control and variation due to differences in the initial 24 hour value for each chick is considerably reduced.

The distribution by RPR of chicks injected with TSH tends to be skewed, particularly at the higher dose levels. The arithmetic average of the individual RPRs thus tends to be dominated by the high values for one or two refractory chicks. To minimize the effect of such chicks on the precision of the assay we have treated the results as if the distribution were log normal rather than normal and have computed geometric rather than arithmetic means.

The computation of the geometric mean and its 68% confidence limits is illustrated using the data in Table 1, daily dose of 13.3 mu after three days.

RPR	*Log RPR*
33	1.519
19	1.279
8	0.903
12	1.079
21	1.322
12	1.079

Arithmetic mean of logs = 1.1968
Geometric mean = antilog 1.1968 = 15.7
Maximum − Minimum = 1.519 − 0.903 = 0.616
Standard error of mean of logs = 0.616/6 = 0.1027
68% confidence limits on arithmetic mean of logs = $1.1968 \pm .1027$
$= 1.0941$ to 1.2995
68% confidence limits on geometric mean = antilog 1.0941 to antilog 1.2995
$= 12.4$ to 19.9
Approximate standard deviation around mean $= \left(\frac{19.9-12.4}{2}\right)\sqrt{6} = 9.2$

Summary results of 15 experiments on the effect of differing doses of TSH on I^{131} depletion are shown in Table 2. Shown for each experiment are (a) the slope of the straight line connecting RPR with the logarithm of dose, (b) the estimated standard deviation at two levels of RPR—50% and 20% and (c) the calculated index of precision λ, defined as the ratio for the standard deviation to the slope at these same two levels of response. Six of the fifteen experiments involved three daily injections of TSH, seven involved two and two involved only one. Each experiment has been so analyzed as to permit a comparison of the precision obtained after each of the number of daily injections used.

The analysis of these results is complicated by the fact that responses are not equally variable at all dose levels. Analysis of the accumulated experience indicates that the standard deviations are at a maximum for RPR in the neighborhood of 50% and fall off as the RPR departs in either direction from this level. If we denote RPR by Y, then the standard deviation at a level of response Y is approximately proportional to $\sqrt{Y(100-Y)}$. The slopes shown in Table 2 are obtained from the weighted least squares straight lines connecting RPR to log dose, where the weights according to Bliss (9) are $n/Y(100-Y)$. The standard deviations shown in the table are the maximum likelihood estimates and are given for a 50% level of response by the formula

$$(\text{st. dev.})^2 = \left\{\frac{\Sigma\, n_i s_i^2/Y_i(100 - Y_i)}{\Sigma\, n_i}\right\} 50(100 - 50)$$

where i stands for a particular dose level and n, s, and Y are respectively the number of chicks, the approximate standard deviation and RPR at that dose level. For the 20% level of response the formula is the same, but

TABLE 2. SLOPE, STANDARD DEVIATION AND INDEX OF PRECISION, λ, FOR DIFFERENT EXPERIMENTS, FOR ONE, TWO, AND THREE DAYS INJECTION. CHICKS WERE FASTED UNLESS OTHERWISE INDICATED. ALL WERE INJECTED WITH PTU PLUS 8 μG. THYROXINE. TSH USED WAS C_5I_5 UNLESS OTHERWISE INDICATED

Date	No. inj.	Slope (RPR/log. dose)	Standard deviation at RPR of		Index of precision (λ) at RPR of	
			50%	*20%*	*50%*	*20%*
3/8	3	71.0	17.8	14.3	.251	.201
	2	66.2	13.7	10.9	.207	.165
	1	47.7	13.2	10.5	.277	.221
3/15	3	69.9	14.9	11.9	.213	.169
	2	60.3	13.3	10.7	.221	.177
	1	46.1	10.2	8.2	.221	.177
3/15[1]	3	75.6	18.3	14.7	.241	.193
	2	72.2	20.6	16.5	.285	.229
	1	55.2	14.6	11.7	.265	.213
3/22	3	107.2	12.6	10.0	.118	.094
	2	97.0	11.3	9.1	.116	.093
	1	68.5	8.1	6.4	.126	.099
4/29	3	56.3	17.9	14.4	.318	.256
	2	49.8	13.4	10.8	.275	.221
	1	29.8	10.5	8.4	.352	.281
6/11	3	61.0	12.0	9.6	.197	.157
	2	62.8	14.7	11.7	.234	.187
	1	41.6	11.4	9.2	.274	.221
4/13	2	77.2	12.9	10.3	.167	.133
	1	40.5	8.5	6.8	.210	.168
4/20	2	89.8	22.0	17.6	.245	.196
	1	64.0	12.4	10.0	.194	.156
5/4	2	87.1	14.2	11.3	.163	.129
	1	53.9	12.8	10.3	.238	.191
5/11	2	78.7	14.5	11.6	.184	.148
	1	60.3	15.7	12.5	.261	.208
5/18	2	49.6	32.9	26.4	.662	.532
	1	42.6	29.3	23.5	.687	.552
5/25	2	92.2	11.8	9.5	.128	.103
	1	50.2	13.5	10.8	.269	.215
6/1	2	75.9	21.6	17.3	.285	.228
	1	64.6	23.4	18.7	.362	.289
3/29	1	44.6	13.2	10.5	.296	.236
3/29[2]	1	47.6	14.0	11.2	.294	.236

[1] Fed low I diet (C_2-KI).
[2] U.S.P. Standard.

20 is substituted for 50. The standard deviations and indexes of precision for 20% RPR are therefore 0.8 of those for an RPR of 50%.

Table 3 summarizes the data of Table 2. The slope is approximately the

TABLE 3. AVERAGE VALUES OF SLOPE AND INDEX OF PRECISION, λ, FOR ONE, TWO, AND THREE DAILY INJECTIONS AT TWO DIFFERENT LEVELS OF RESPONSE (RPR). DATA FROM TABLE 2

First six experiments	Number of daily injections		
	3	2	1
Slope (RPR/10 fold)	73.5 ±8.5	68.0 ±7.9	48.2 ±6.4
λ: at RPR of 50%	0.22 ± .03	0.22 ± .03	0.25 ± .04
λ: at RPR of 20%	0.18 ± .02	0.17 ± .02	0.20 ± .02
All experiments			
Slope (RPR/10 fold)	73.5 ±8.5	73.8 ±3.6	50.5 ±2.6
λ: at RPR of 50%	0.22 ± .03	0.24 ± .04	0.29 ± .04
λ: at RPR of 20%	0.18 ± .02	0.21 ± .03	0.22 ± .02

same at 2 and 3 days, but much greater than at day one. As indicated by the index of precision, λ, there is no important difference between the two day and three day assay. The two day assay is somewhat more precise than that for one day, the values of λ at an RPR of 50% being 0.24 and 0.29, respectively. The standard error of this difference, estimated from the paired comparison, is only 0.015, so that the difference between the λ's, although seemingly small, is statistically significant. The difference is not as large as one would expect from comparison of the slopes alone, because the standard deviation at comparable RPRs is also smaller for the one day assay. The percentage increase in the number of chicks required in a one day assay designed to have the same precision as a two day assay can be calculated from the ratio of the λ's, namely $(0.29/0.24)^2 - 1$. Thus, one requires 46% more chicks in a one day assay. In view of the fact that the one day assay requires less work done on each chick and one day less time, the increase in the required number of chicks does not appear excessive. For many purposes therefore the one day assay can be recommended as the one most likely to give results of required precision with the least amount of experimental effort.

The total number (n) of chicks[3] needed to attain a selected accuracy of determination is given by the formula $n = (2\lambda/s_M)^2$ where s_M is the selected standard error of log potency. If the latter is chosen as 0.08, corresponding to an error of ±20% on the estimate of potency (antilog 0.08 − 1), then 40 chicks are needed with a λ of 0.25 and 26 with a λ of 0.20. If s_M corresponding to an error of ±50% is selected, then 14 chicks are needed with a λ of 0.25 and 9 chicks with a λ of 0.20.

Effects of auxiliary substances and food on iodine depletion

With No TSH:

Table 4 shows the effect of various substances on I^{131} depletion in the chick in the absence of exogenous TSH. The data for the 72 hour period

[3] This is not the number of chicks at any one dose level, but the total number, both for standard and unknown and for all dose levels. Thus, n = 16, means 4 chicks at each of two dose levels for standard and unknown.

TABLE 4. EFFECT OF THYROXINE AND PROPYLTHIOURACIL ON IODINE DEPLETION IN THE FED AND IN THE FASTED CHICK

Auxiliary treatment	Daily dose	24 hr. RPR %	48 hr. RPR %	72 hr. RPR %	Approx. ½ life days	Equivalent[1] Endogenous TSH mu/day
Fasted						
Uninjected	—	96 ± 2.4	89 ± 3.2	85 ± 2.8	13	(2?)
Thyroxine	8 μg.	98 ± 1.5	94 ± 1.4	93 ± 2.8	28	<2
PTU	5 mg.	82 ± 3.5	67 ± 4.1	55 ± 5.0	4	4
Solution A (Thyroxine + PTU)	8 μg. + 5 mg.	97 ± 2.5	94 ± 2.0	91 ± 2.3	24	<2
Fed[2]						
Uninjected	—	93 ± 1.8	81 ± 1.5	64 ± 2.6	5.0	3
Thyroxine	8 μg.	99 ± 1.5	94 ± 2.8	86 ± 1.3	13	(2?)
PTU	5 mg.	72 ± 5.8	52 ± 6.5	33 ± 7.9	2	8
Solution A (Thyroxine + PTU)	8 μg. + 5 mg.	92 ± 1.9	88 ± 3.6	78 ± 3.5	8	2

[1] On basis of data from fasted chicks injected with A and TSH.
[2] Low iodine diet (C_2-KI) ad libitum.

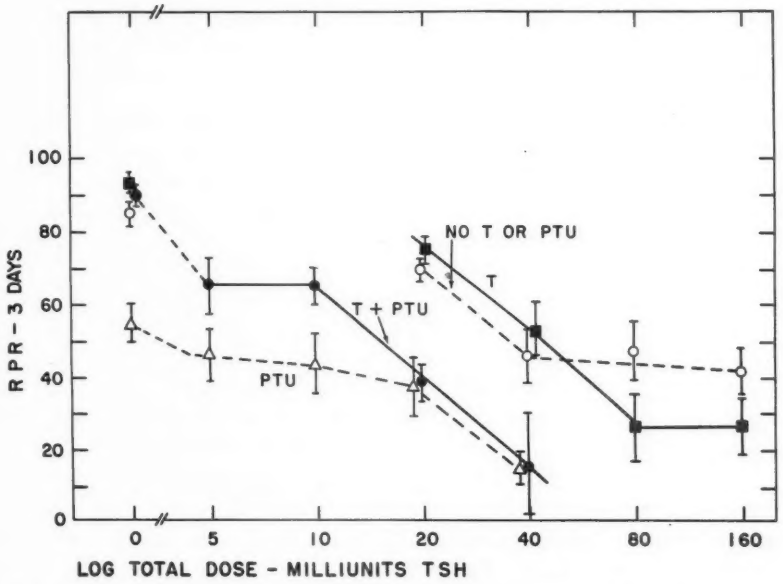

FIG. 1. Effect of 5 mg. of PTU and 8 μg. of thyroxine, separately and together, on the depletion of I^{131} with various amounts of TSH. Only the RPR after 3 daily injections is shown. Five chicks/group.

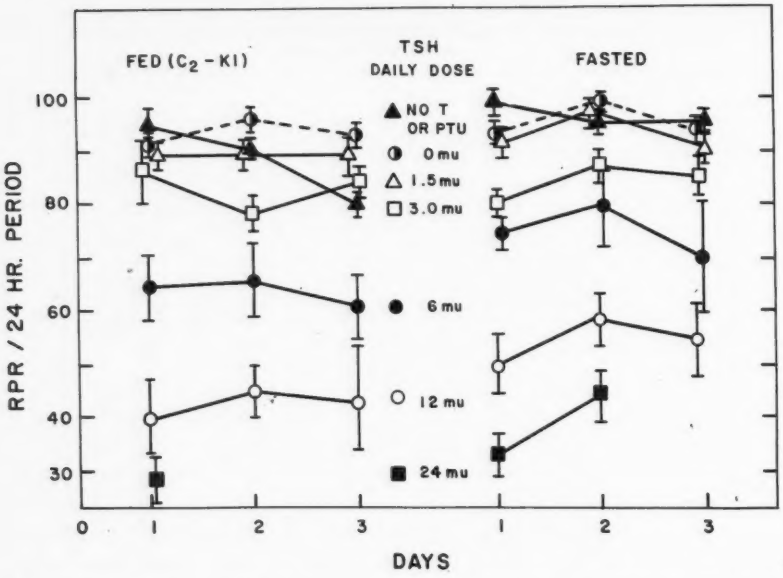

FIG. 2. Showing the constancy of RPR for each 24 hour period of injection. All chicks received 5 mg. of PTU and 8 μg. of thyroxine daily, except the uninjected groups, ▲. Five chicks per group.

on the fasted chicks are also plotted in Figure 1 as the control data. Thyroxine, alone, almost completely stops depletion in fasted chicks and partially inhibits depletion in fed chicks. PTU, alone, produces a marked depletion in both fasted and fed chicks. When given with PTU, thyroxine reduces the rate of depletion below that of uninjected chicks, but there is still slightly greater depletion than when thyroxine alone is injected.

Food is seen to increase the rate of depletion under all four conditions of treatment. Especially interesting is the increased rate of depletion with each day in the case of uninjected chicks when fed. This increasing rate of depletion is best seen graphically plotted in Figure 2. This increasing rate of depletion would suggest an increasing output of endogenous TSH under the stimulus of food, but other possibilities exist, such as increased excretion of iodine. This depletion with feeding is accompanied by a parallel decreasing I^{127} concentration in the thyroid (unpublished). The synthetic diet used (casein, gelatin, dextrose and corn oil) can hardly be considered to contain any goitrogenic substance. Hence, alimentation itself is a factor in stimulating release of endogenous TSH from the pituitary or elimination of iodine from the body, or both.

Conversely, this means that fasting slows the rate of depletion of I^{131}. In fact, the biological half life of I^{131} is the same (13 days) in fasting un-

injected chicks as in fed chicks injected with thyroxine which blocks endogenous TSH release.

With TSH:

The effect of thyroxine and PTU upon the dose-response curve after 3 daily injections of TSH is shown graphically in Figure 1. Several effects are apparent.

1. When PTU is injected, the dose of TSH required to produce a given amount of depletion is decreased by a factor of at least 2.
2. When no PTU is given, the RPR will not fall in 3 days below 30–40%, even when doses up to 160 mu of TSH are injected. PTU administration permits depletion to an RPR below 10% thereby extending the useful range of dose by a factor of about 2.
3. The depletion with PTU at 0 dosage of TSH (RPR = 55%) may be considered to result from endogenous TSH production and is equivalent to 4 mu of TSH per day, or a total dosage of 12 mu of TSH over 3 days (Table 4). The dose-response curve for PTU alone becomes almost identical with that for thyroxine plus PTU, if this 12 mu representing endogenous TSH, is added to the amount of exogenous TSH injected at each dosage interval.
4. The results obtained with thyroxine alone and especially with the combined dosage of thyroxine and PTU (Table 4 and Figure 1) indicate that thyroxine inhibits endogenous TSH production.

Figure 3 is a log dose-response plot of the 3-day data shown in Figure 2. The log dose-response curves for fed and fasted chicks, both given auxiliary

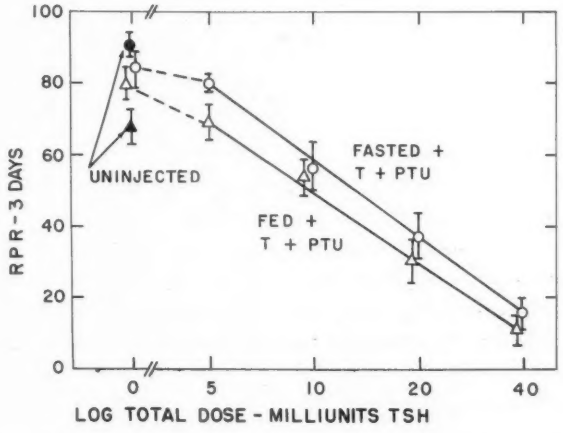

FIG. 3. Effect of feeding on depletion of I^{131} after 3 daily injections. Data for the third day from Fig. 2 plotted against dosage of TSH.

treatment of thyroxine and PTU, are compared. It will be noted that the RPR is consistently higher for the fasted chicks, for every dose of TSH given, as well as for the zero dose. The two points labelled uninjected are those obtained with chicks given no TSH and no auxiliary solution. If feeding caused an increased release of a fixed amount of endogenous TSH the dose-response curves in Figure 3 would be expected to approach each other as the dose of TSH increases. Parallelism would indicate that the greater depletion in fed chicks is due to increased elimination of iodine from the body accompanying the excretion of digested food products. In view of the standard errors at each point, the data are consistent with both the hypothesis of parallelism and of convergence.

In summary, PTU increases both the sensitivity and the range of applicability of the dose-response curve. Because PTU causes iodine depletion equivalent to the endogenous production of 4 mu/day of TSH, even in the fasted chick, it is necessary to give thyroxine as a second auxiliary substance to counteract this effect.

Effect of number of days of injection on dose-response curve

It has been a regular finding when the auxiliary solution is used, that repeated daily injections of the same dosage of TSH results in the same average RPR for each 24 hour period, so long as the dosage does not exceed that needed to produce an RPR of less than 30%. This is a dosage of about 15 mu. An RPR of about 25% has been found to be the limit of depletion approached at high doses of TSH after 24 hours. This constancy of the average RPR per day for both fed and fasted chicks is shown in Figure 2. Two characteristics of the present bioassay method are explained by the constancy of the daily RPR, namely,

(1) The apparent steepness of the two and three day dose-response curves as compared with the one day curve, and
(2) The amount of depletion with a given amount of TSH is about the same, whether this amount is given in one, two, or three daily injections.

To show this, we have constructed theoretical dose-response curves for the one, two, and three day assay by assuming

(a) that the two day dose-response curve is linear with a slope of 75 for a ten-fold dilution (Table 3).
(b) that the average daily RPR is constant (Fig. 2). This implies that at any given daily dose of TSH, the three day RPR will be the cube and the two day RPR the square of the one day RPR. Both assumptions are based on experience.

We show in Figure 4 the theoretical one, two, and three day dose-response curves that follow from these assumptions, plotted against the

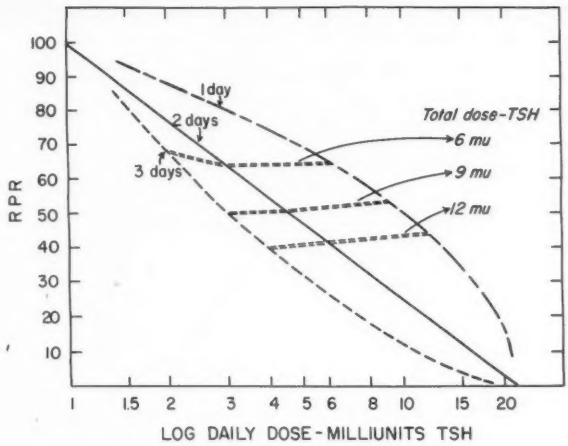

FIG. 4. Theoretical curves showing how RPR varies with dosage of TSH at 1, 2 and 3 days when chicks are injected concurrently with solution A. That RPR depends on total dose of TSH is also indicated.

logarithm of daily dosage of TSH and in Figure 5 the same curves plotted against the logarithm of total dosage of TSH. The equations of the three curves in Figure 4 are:

$$RPR_1 = 100\sqrt{1-0.75 \log x}$$

$$RPR_2 = 100[1-0.75 \log x]$$

$$RPR_3 = 100[1-0.75 \log x]^{3/2}$$

where x = milliunits of TSH/day

It will be noted that, strictly speaking, of the three dose response curves, only that for the two day assay is linear, while those for the one and three day assays show a certain curvature but in opposite directions. This curvature is of course too slight to be easily detected experimentally, especially because there is a small iodine depletion in all control groups down to an RPR of 90–95 in one day and 85–90 in 2 to 3 days. It also follows from this this theoretical treatment (Fig. 5) that the sensitivity is best at the one day dosage level. Thus, a total dose of 2 mu could be detected in 1 day, but not if spread over 2 or 3 days. The average slope of the theoretical 1 day curve in the RPR range of 50–90 (which is where most of our one day assays have been conducted) is about 55, or almost one-third below that for the two day curve. On the other hand, the average slope of the three day curve in the RPR range of 10–75 is only slightly above 75. Both results agree closely with what was actually found (Table 3).

How different will the RPR corresponding to a given total TSH dosage be when the interval of time over which this dosage is given is varied?

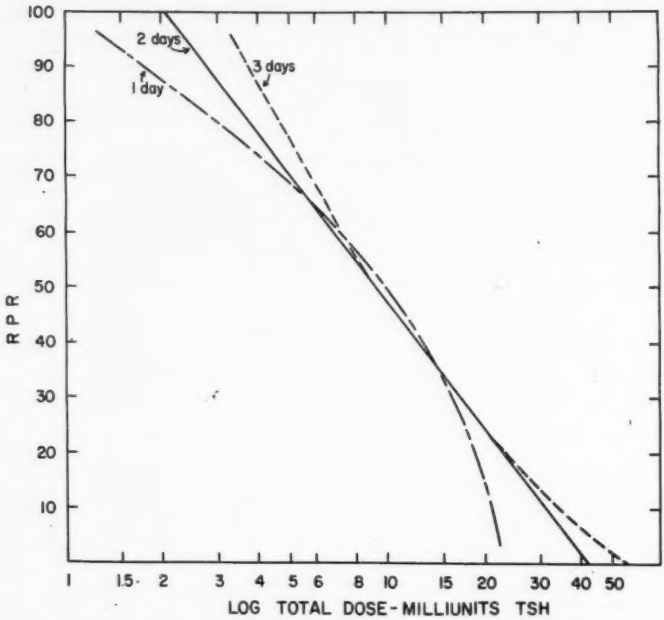

FIG. 5. Same theoretical curves as in Fig. 4 plotted against total dose of TSH. Shows dependence of RPR on total dose of TSH whether given in 1, 2 or 3 daily injections.

To show this in Figure 4, the RPR for a given daily dosage on the one day curve has been connected with the RPR for one-half that daily dosage on the two day curve and with the RPR for one-third that dosage on the three day curve. The corresponding RPRs are essentially constant at the three dosage levels shown. To show this more fully, we have replotted the theoretical data of Figure 4 against total dosage in Figure 5. Over a very considerable range of dosage, the amount of depletion, as measured by total RPR, is almost independent of the number of days over which this dosage is spread and dependent solely on the amount of TSH injected.

Parallelism of dose-response curves

In Figure 6 the 2-day RPR at three different dose levels obtained for the U.S.P. standard and a 75% acetone insoluble fraction, 95-E, from bovine pituitaries are compared in a simultaneous assay. It will be noted that both dose-response curves are essentially linear and parallel. The one and three day dose-response curves yielded by this experiment were also nearly linear and parallel.

Cross-over design

The constancy of the daily RPR when the same daily dosage is given three days in succession suggests the possibility of using a cross-over de-

sign. In such a design one would give a standard and unknown preparation on successive days to the same chick. We have tried such designs, but do not recommend them for two reasons: (a) to avoid carryover effects, it is necessary to restrict the dosage given on the first day to an inconveniently narrow range, and (b) even when such restrictions are made, the design results in no increase in precision over the more usual design.

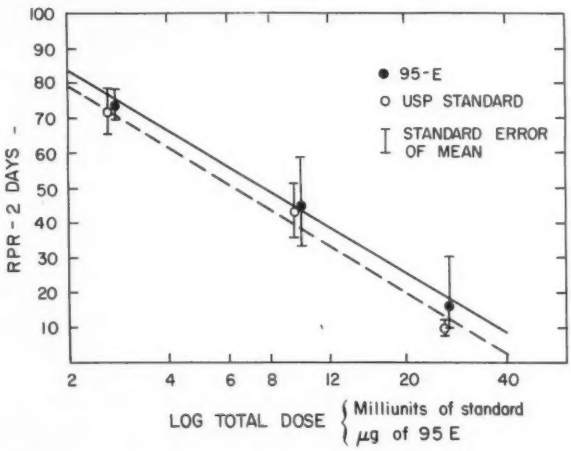

FIG. 6. Typical bioassay data after 2 injections showing parallelism. Solution A given concurrently with TSH. Five chicks/group.

DISCUSSION

With large doses of TSH in the absence of PTU (Fig. 1) a depletion limit at about 30% RPR was found even after 3 injections. The data of Gilliland and Strudwick (3), who do not use PTU in their method, confirm the existence of this limit since their dose-response curve stops at about 60% depletion or an RPR of 40%. This limit probably is related to the processes of reutilization of iodine. A comparison of the index of precision, for Gilliland and Strudwick's data ($\lambda = 0.4$ to 0.5) with that found herein ($\lambda = 0.2$ to 0.25) shows an increased accuracy perhaps due to the action of PTU.

Much of the success in this assay method depends upon having available day old chicks that must have an uptake of I^{131} greater than 15% at 24 hrs. Chicks from most vendors have less than 15% uptake. A search for a source of chicks with high uptake is worthwhile. Apparently Gilliland and Strudwick had chicks with low uptake for they had to use a 5 to 10 times larger dose of I^{131} than used herein and wait three days for the body background to decrease sufficiently for the *in vivo* thyroid counts to become significant. This longer time interval necessitates feeding the chicks a low iodine diet which eliminates the advantage of fasting.

SUMMARY

Day-old chicks having more than a 15% uptake of I^{131} by the thyroid were injected with 3 μc. of I^{131}. Over the next three days counts are made *in vivo* and TSH and an auxiliary solution containing 8 μg. of thyroxine and 0.5 to 5 mg. of propylthiouracil are injected. Propylthiouracil facilitates depletion and increases the sensitivity and accuracy of the assay. The extent of the depletion of I^{131} for each of the three days was compared. The amount of depletion within certain limits of total dosage was found to be the same whether the dosage was given over 1, 2, or 3 days. A linear relation between log dose and the extent of depletion was found with an index of precision, λ, of about 0.20.

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ACUTE EFFECTS OF TRANQUILIZING DRUGS ON THE ANTERIOR PITUITARY-ACTH MECHANISM

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THE use of the tranquilizing agents in the therapy of the psychiatric patient has promoted considerable interest among investigators concerning the mechanism of action of these drugs. Reported success in sedating disturbed individuals prompted the present investigation to study the effects of the tranquilizing drugs on the anterior pituitary-cortical mechanism. Recent work in this laboratory indicates that there is a high degree of correlation between plasma 17-hydroxycorticosteroid (17-OH-CS) levels and clinically estimated levels of anxiety in patients awaiting elective thoracic surgery (1). Similar plasma 17-OH-CS elevations were associated with experimentally conditioned "anxiety" and avoidance responses in the rhesus monkey (2). Since the tranquilizing drugs can suppress "anxiety," clinically and in the experimental animal, it might be expected that these drugs may depress anterior pituitary activity resulting in decreased levels of 17-OH-CS in peripheral blood. However, Egdahl *et al.* (3) found elevations of 17-OH-CS in the adrenal vein blood of the dog following reserpine administration. Tui *et al.* (4) found elevations of 17-OH-CS in peripheral blood of schizophrenic patients in response to reserpine and depression following chlorpromazine administration. Since these authors state that the study was random with respect to duration of medication and drug administration, it seemed worthwhile to us to carry out a more rigidly controlled series of experiments to investigate the acute effects of these agents.

It is therefore, the purpose of this investigation to study the effects of reserpine and chlorpromazine on the pituitary-adrenal cortical system and to compare these data with the effects of a CNS depressant, pentobarbital and ACTH. It is felt that these studies may serve as an indirect measurement of the central action of these two classes of agents, particularly in the hypothalamic-hypophyseal area.

METHOD

Adult monkeys, Macaca mulatta, male and female, ranging in weight from 3–5 kg. were caught with a net and restrained on a table while blood was drawn from the saphenous vein. About 8 ml. of blood was drawn at each bleeding and the 17-OH-CS

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concentration was determined on the plasma using the Nelson-Samuels Method (5, 6).

The diurnal variation was established on 7 monkeys by determining the 17-OH-CS level at 0, 2 and 6-hour intervals where the zero time was 9:00 A.M. This group of animals was used for all subsequent experiments to be described. Each individual animal was used on an average of every 10 days. All drugs used were administered by single intravenous injection immediately following the zero hour bleeding and blood samples were then drawn at 1, 2 and 4 hours for 17-OH-CS determinations.

Nembutal[1] (30 mg./kg.) was given to 5 monkeys. All the animals remained anesthetized for about 4 hours. ACTH (Armour) was taken up in isotonic sodium chloride. Each of 6 animals was given 16 mg./kg.; 4.0 mg./kg.; and 0.05 mg./kg. on different days. Reserpine (Ciba, Serpasil) was made up in saturated citric acid with moderate heat and diluted with distilled water to give a final solution containing 1 mg./ml. of reserpine in 0.75% citric acid. Doses administered on different days were as follows: 0.05 mg./kg.; 0.10 mg./kg.; 0.25 mg./kg.; 0.50 mg./kg.; and 1.0 mg./kg. The latter dose was given to 6 different monkeys while each of the other doses was tested on 3 animals, Chlorpromazine was studied in less detail. The 17-OH-CS response to chlorpromazine was evaluated in one animal after 0.5 mg./kg.; 2.5 mg./kg.; and 5.0 mg./kg. and in a second monkey after 1.0 mg./kg. and 5.0 mg./kg.

RESULTS

Table 1 shows peripheral blood levels of 17-OH-CS in normal monkeys over a six-hour period of time starting at 9:00 A.M. This time span represents that part of the day during which all experimental procedures were studied in these animals. Normal values were determined on two different occasions, and the last column of the table shows the average value for each animal as well as the mean average values of the entire group. The data show a decline in concentration of the 17-OH-CS from 35 ug.% in the A.M. to 26 ug.% in the P.M. This is compatible with the diurnal variation exhibited by other laboratory animals (7) and by humans (8). Failure of the 17-OH-CS levels to rise in the two-hour samples insures that there was no adrenal response of the animals to handling.

Measurement of peripheral blood levels of 17-OH-CS after various dosages of ACTH serves as a point of reference for anterior pituitary-adrenal cortical activity. The data shown in Table 2 give the concentrations of 17-OH-CS in peripheral blood at 1, 2, and 4 hours following various doses of ACTH. The peak responses for the smallest dose, 0.05 mg./kg., occurred at one hour. The intermediate dose, 4.0 mg./kg., elicited the highest level at two hours. With both these dose schedules the 17-OH-CS level is approaching normal at four hours. When the largest dose, 16.0 mg./kg., was given the peak response also occurred at two hours but maintained a plateau for four hours. However, the rate at which the 17-OH-CS concentration increased during the first two hours was the same after both the 4.0 mg./kg., and the 16.0 mg./kg. doses of ACTH. This indicates that there is a rate limit beyond which the adrenal cortex cannot be further stimulated. It appears that the maximal rate of stimulation results from

[1] Pentobarbital Sodium, Abbott.

TABLE 1. NORMAL PLASMA LEVELS OF 17-HYDROXYCORTICOSTEROIDS IN MONKEY (MACACA MULATTA)

Monkey	Time* (hr.)	17-OH-CS (μg./100 ml. plasma)		
		Experiments		Av.
		1	2	
4	0	33	33	33
	2	33	28	31
	6	23	21	22
5	0	28	40	34
	2	36	44	40
	6	17	45	31
9	0	39	45	42
	2	39	36	38
	6	29	33	31
10	0	23	24	24
	2	26	27	27
	6	17	28	23
11	0	47	31	39
	2	45	26	36
	6	26	4	15
12	0	44	44	44
	2	29	44	37
	6	9	32	21
13	0	27		27
	2	30		30
	6	37		37
Mean	0			35
	2			34
	6			26

* 0 Hr. = 9:00 A.M.

the 4.0 mg./kg. dose while larger doses exhibit a more prolonged effect. These data are shown graphically in Fig. 1.

Monkeys to which Nembutal was administered showed, in all cases, an anesthetic response which lasted for four to five hours. The 17-OH-CS reaction of Nembutal was, in general, one of depression in concentration of peripheral levels. The data over the course of the four hours during which the animals were studied are given in Table 3. Four of the 5 animals showed a decrease in 17-OH-CS levels. Animal No. 12 showed an anomalous response—a rise in 17-OH-CS level at one hour followed by a slight fall at four hours after the administration of Nembutal.

Doses of reserpine from 0.05 mg./kg. to 1.0 mg./kg. caused increases in the peripheral content of 17-OH-CS. The data are given in Table 4. With the two lowest doses, i.e., 0.05 and 0.10 mg./kg., the peak of 17-OH-CS response occurred at two hours and the levels were approaching normal by four hours. With the higher doses, 0.25, 0.50, and 1.0 mg./kg., the 17-OH-CS concentrations were still rising at four hours. The rate of rise of 17-OH-

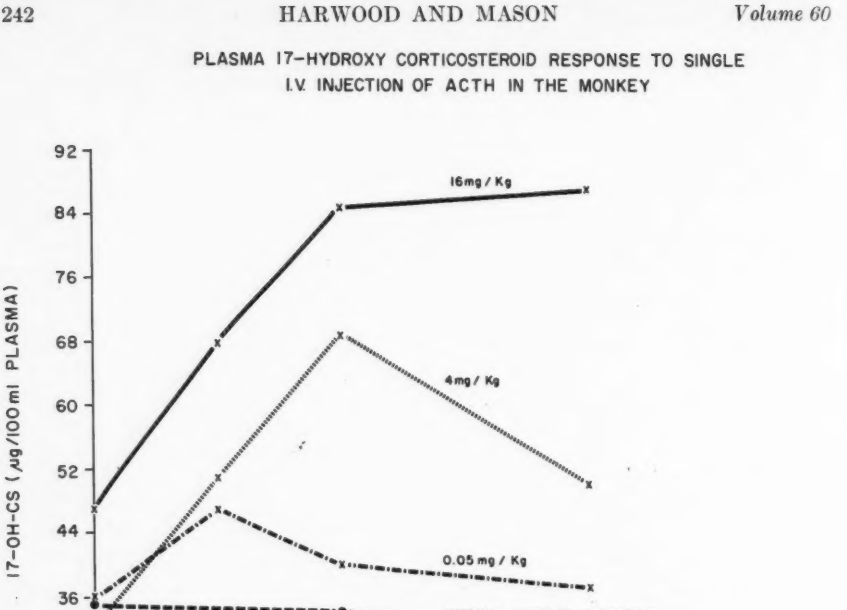

FIG. 1

CS concentration after the highest dose of reserpine (1.0 mg./kg.) is comparable to that elicited by the 4.0 and 16.0 mg./kg. doses of ACTH. It is apparent that reserpine administration results in maximal stimulation of the adrenal cortex.

Chlorpromazine, the other member of the group of tranquilizing drugs investigated in these experiments, was studied in less detail than reserpine. However, it is evident from the data given in Table 5 that chlorpromazine has the ability to increase the circulating levels of the 17-OH-CS. Endocrine response increased with dose up to the 2.5 mg./kg. dose but a 5.0 mg./kg. dose on two occasions was less effective in raising the levels of the 17-OH-CS.

DISCUSSION

It is clear that Nembutal and reserpine produce effects on the anterior pituitary-adrenal cortical system which are quite different. Nembutal results in lower peripheral blood levels of 17-OH-CS and reserpine in elevated levels. The animals appear to have a wide range of sensitivity to Nembutal as is seen by the variation in 17-OH-CS concentration but the

TABLE 2. PERIPHERAL PLASMA LEVELS OF 17-HYDROXYCORTICOSTEROIDS FOLLOWING SINGLE INTRAVENOUS INJECTION OF ACTH IN THE MONKEY (MACACA MULATTA)

Monkey No.	Dose (mg./kg.)	17-OH-CS (μg./100 ml. plasma)			
		0 Hr.*	1 Hr.	2 Hr.	4 Hr
4	0.05	43	56	50	35
5	0.05	38	52	37	38
9	0.05	35	45	33	40
10	0.05	31	33	35	31
11	0.05	33	48	50	53
13	0.05	36	48	33	23
Mean		36	47	40	37
4	4.0	34	55	74	41
5	4.0	34	47	52	38
10	4.0	24	52	75	44
11	4.0	38	59	87	87
13	4.0	29	41	59	42
Mean		32	51	69	50
4	16.0	42	60	86	98
5	16.0	50	70	81	67
10	16.0	41	60	69	76
11	16.0	50	93	120	106
13	16.0	50	57	67	90
Mean		47	68	85	87

* 9:00 A.M.

average two hour levels after Nembutal administration are significantly different from the two hour levels in the diurnal variation study as evidenced by the t test, $P = 0.05$. Nembutal is known to exert a differential depressant effect on various parts of the central nervous system (9). In these animals the decrease in output of adrenocorticotrophic hormone may have been due to depression of activity in areas normally stimulating adeno-hypophyseal secretion.

TABLE 3. PERIPHERAL PLASMA LEVELS OF 17-HYDROXYCORTICOSTEROIDS FOLLOWING SINGLE INTRAVENOUS INJECTION OF NEMBUTAL IN THE MONKEY (MACACA MULATTA)

Monkey No.	Dose (mg./kg.)	17-OH-CS (μg./100 ml. plasma)			
		0 Hr.*	1 Hr.	2 Hr.	4 Hr.
5	30	32	30	12	—
9	30	30	29	22	19
10	30	25	—	21	13
11	30	35	30	21	7
12	30	32	40	30	25
Mean		31	32	21	16

* 9:00 A.M.

TABLE 4. PERIPHERAL PLASMA LEVELS OF 17-HYDROXYCORTICOSTEROIDS FOLLOWING SINGLE INTRAVENOUS INJECTION OF RESERPINE IN THE MONKEY (MACACA MULATTA)

Monkey No.	Dose (mg./kg.)	17-OH-CS (μg./100 ml. plasma)			
		0 Hr.*	1 Hr.	2 Hr.	4 Hr.
4	1.0	25	32	32	53
5	1.0	39	46	55	69
9	1.0	31	47	56	63
10	1.0	28	57	67	63
11	1.0	13	53	148	105
13	1.0	21	43	54	72
Mean		26	46	69	71
5	0.5	24	35	35	60
10	0.5	18	31	42	56
13	0.5	33	56	54	79
Mean		25	41	44	65
9	0.25	28	40	41	63
10	0.25	37	40	45	50
11	0.25	52	70	87	90
Mean		39	50	58	68
5	0.10	33	48	50	38
10	0.10	22	28	—	32
13	0.10	27	38	52	44
Mean		27	38	51	38
5	0.05	28	32	50	38
11	0.05	35	38	32	31
13	0.05	41	56	66	44
Mean		35	42	49	38

* 9:00 A.M.

The clinical behavior of the monkeys following the three highest doses of reserpine was one of marked sedation following a latent period of about two hours. With the 1.0 mg./kg. dose of reserpine the animals remained in a stupor-like condition unless aroused by some strong tactile stimulus. The

TABLE 5. PERIPHERAL PLASMA LEVELS OF 17-HYDROXYCORTICOSTEROIDS FOLLOWING SINGLE INTRAVENOUS INJECTION OF CHLORPROMAZINE IN THE MONKEY (MACACA MULATTA)

Monkey No.	Dose (mg./kg.)	17-OH-CS (μg./100 ml. plasma)			
		0 Hr.*	1 Hr.	2 Hr.	4 Hr.
4	0.5	35	43	55	49
4	2.5	39	55	70	39
4	5.0	25	40	46	49
5	1.0	27	34	46	48
5	5.0	44	37	37	39

* 9:00 A.M.

effect of the tranquilizing agent and its mode of action on the central nervous system appear quite different from that of the CNS depressant. The specific sites of action of reserpine have not been established. These data on the plasma 17-OH-CS levels would indicate an increased output of adrenocorticotrophin. Recently Brodie (10) has postulated that reserpine acts by release of serotonin which may function as a neurohumoral agent. Whatever the mechanism of action of reserpine on the CNS its effects on the hypothalamic-pituitary system, as shown by the 17-OH-CS data, are related to the amount of the drug administered.

The dose-response kinetics of chlorpromazine differ from that of reserpine. On a weight basis chlorpromazine is not as potent as resperpine in stimulating the pituitary-ACTH system. The two lower doses of chlorpromazine result in graded 17-OH-CS response. The highest dose was less stimulating in terms of 17-OH-CS than the intermediate dose. The experiments reported here were not designed to explain this dose-response behavior. However, reversal of effect with change in dose is not restricted to the pituitary-adrenal cortical system. Rinaldi and Himwich (11) reported stimulation of electrical activity from high doses of chlorpromazine and depression of electrical activity from low doses in the reticular formation of the rabbit.

Chlorpromazine may act by selectively affecting combinations of stimulator or inhibitor areas in the CNS. Whatever specific sites are involved in the drug action, it is apparent from the data that the net effect of low doses is stimulation of the anterior pituitary-ACTH system while high doses fail to stimulate this system.

SUMMARY

Peripheral blood concentrations of 17-OH-CS undergo a diurnal variation in the monkey, ranging from 35 μg./100 ml. plasma in the A.M. to 26 μg./100 ml. plasma in the P.M. Nembutal depresses the 17-OH-CS concentration in plasma while the tranquilizing drugs, reserpine and chlorpromazine, elevate this level. Using the 17-OH-CS response to ACTH as a standard of reference, it appears that reserpine results in maximal stimulation of the adrenal cortex.

Acknowledgment

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MORPHOLOGICAL OBSERVATIONS ON THE ADRENAL CORTEX OF THE LABORATORY RAT[1]

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INVESTIGATIONS of adreno-cortical morphology have been overshadowed by extensive experimental and clinical reports.

The results of many such experiments on the adrenal cortex of the rat have been considered by many workers applicable to theories of human physiology. Such an attitude may be fallacious, because specific morphological differences exist in the adrenal cortices of various mammals. Since function can be correlated so often with morphology, a thorough consideration of the histology of the adrenal cortex of the rat may be useful at this time. It is the purpose of this paper to describe the normal morphology of the adrenal cortex of the laboratory rat from birth to senility.

Following the classical work of Arnold (1), a vast amount of literature has accumulated on adreno-cortical morphology. Because this literature has been summarized so well in a recent monograph by Bachmann (2), no attempt to review the general literature will be made.

MATERIALS AND METHODS

Adrenal cortices of albino rats from the Sprague-Dawley colony were studied at birth, 3 weeks, 6 weeks, 3 months, 6 months, 1 year and senility. In this paper the term senility refers to animals over 2 years old that have been sterile for at least 6 months. Both adrenals from each of 3 males and 3 females in the above age groups were used in the study of the morphology of the parenchyma and stroma. In order to facilitate the study of the vasculature, a second similar group of animals was perfused with a mixture of NaCl, gum acacia, Bouin's fluid and India ink, a modification of the technic of Koenig, Groat and Windle (3). The tissues were fixed in Bouin's fluid, then dehydrated by alcohols, cleared in xylol, imbedded in paraffin and serially sectioned at 12 μ. They were impregnated with silver according to Gomori's method or stained with either hematoxylin and eosin, van Gieson's stain or a modification of Masson's trichrome stain.

The adrenal glands of 3 other ink-perfused, adult rats were fixed in Bouin's fluid, dehydrated in alcohols and cleared in xylol. These specimens were then manually sectioned as thinly as possible with a razor blade and mounted in balsam. The vascular patterns were studied under a dissecting microscope, and drawings were made.

A three-dimensional analysis of the morphology of the parenchyma, stroma, and vasculature of the adrenal glands from the 87 animals was made by means of glass plate reconstructions. These reconstructions were prepared by projecting serial sections on

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lantern slide cover glasses with a table microprojector (4). The images were traced on the cover glasses with opaque drawing inks of various colors. The outline of the parenchyma was painted with a translucent, colored, acetate lacquer. The serial glass plates were then stacked, and stereograms drawn from them.

A third group of 42 animals was sacrificed, and both adrenals were fixed in calcium-formol. These tissues were washed, infiltrated, and blocked in 25% gelatin. Serial frozen sections were cut at 10 μ on a rotary microtome (5). Sections were stained with sudan IV for the presence of fats.

OBSERVATIONS

A. *Observations on the Vasculature*

1. EXTRACAPSULAR

The arteries to the adrenal glands branch in the periadrenal connective tissue and distribute themselves over the surface of the capsule. Smaller

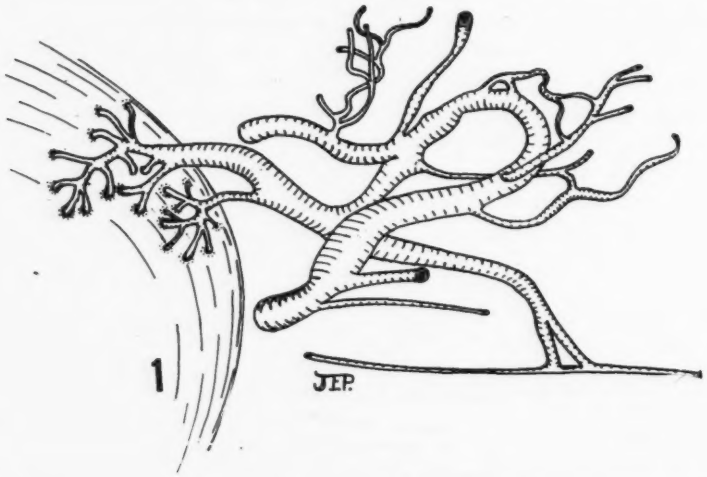

FIG. 1. Reconstruction of arteries branching in periadrenal connective tissue and distributing themselves over surface of capsule.

branches pass to the surrounding connective tissue while the larger ones penetrate the capsule. Figure 1 drawn from a thick, cleared section of adrenal and studied by means of a dissecting microscope illustrates these vessels. These observations confirm the work of Harrison (6) in the rabbit and Lever (7) in the rat.

2. THE SUBCAPSULAR PLEXUS

The arteries that penetrate the capsule form an extensive subcapsular arterial plexus (Figs. 2, 3). This was first described by Bennett and Kilham (8) in the cat and later by Gersh and Grollman (9) in the mouse and rat. This plexus lies parallel to the capsule, between it and the underlying parenchyma. The vessels undergo what Lever (7) has described as a "tree-

like division" down to capillaries in the subcapsular reticulum, and they anastomose among themselves. With few exceptions, the larger arteries of the plexus do not penetrate the parenchyma of the zona glomerulosa but lie between the cellular mass and the capsule.

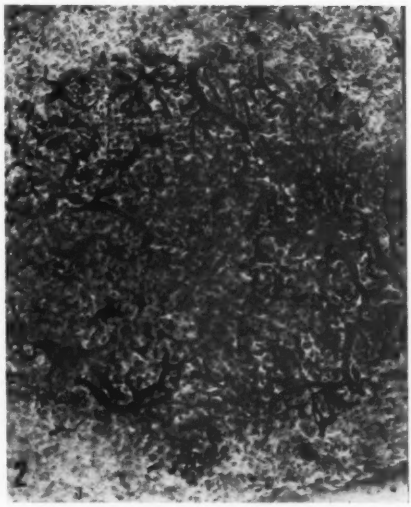

FIG. 2. Tangential section: subcapsular arterial plexus. Ink perfused.

3. VESSELS OF THE ZONA GLOMERULOSA

Vessels arising from the subcapsular plexus penetrate the parenchyma of the zona glomerulosa. A typical radial section shows the sinusoids[3] cut in cross, oblique, and longitudinal sections (Fig. 3). This shows that the vessels within the glomerulosa run through the parenchyma at all angles to the capsule (Fig. 4). Anastomoses among these vessels are common. A study of serial sections and reconstructions of the vasculature after ink perfusion shows that every cell in the zona glomerulosa has at least one facet touching a sinusoid.

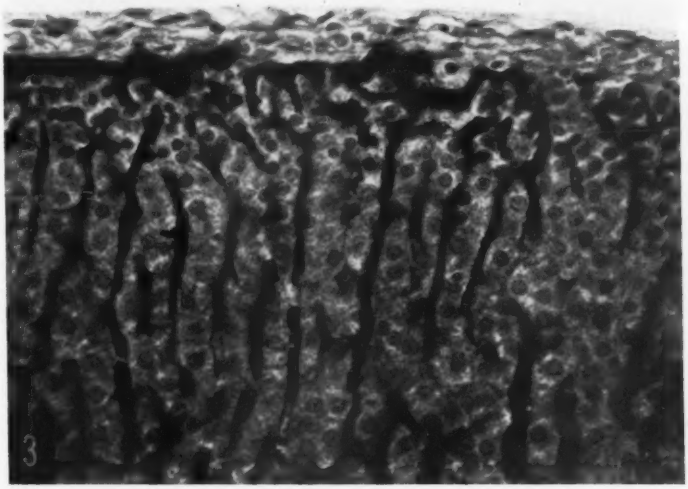

FIG. 3. Radial section: a field of cells interrupted by the subcapsular arterial plexus and the sinusoids of the zona glomerulosa cut at all angles to capsule.

[3] A justification for the use of this word will be found later in the text.

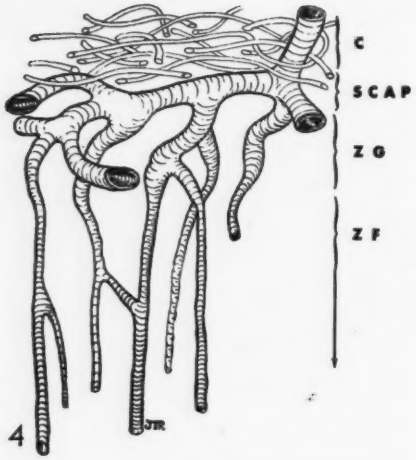

FIG. 4. Steriogram showing relation of capsule, subcapsular arterial plexus and sinusoids of zona glomerulosa running through parenchyma at all angles to capsule.

4. VESSELS OF THE ZONA FASCICULATA

At the junction with the zona fasciculata, the sinusoids of the zona glomerulosa turn and project toward the medulla in a radial direction (Figs. 4 and 5). The vessels are parallel to each other, and anastomoses are frequent. In this zone, the sinusoids have a narrow diameter and contrast sharply with the larger sinusoids of the zona glomerulosa.

Tangential sections through the zona fasciculata show that here too every cell is touched on at least one surface by a sinusoid (Fig. 6). The

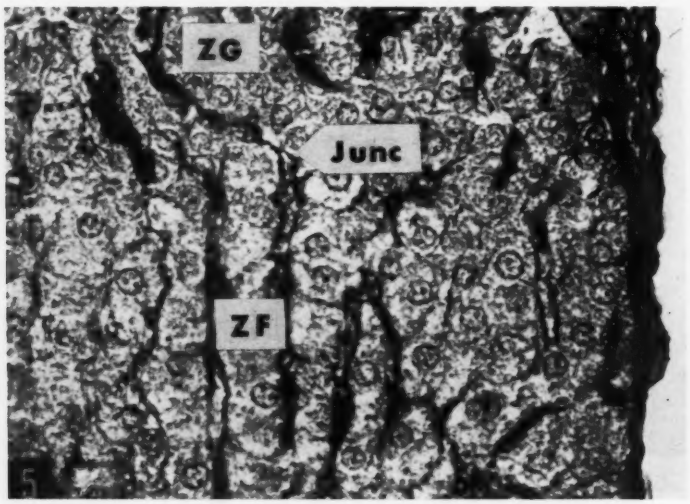

FIG. 5. Radial section: large sinusoids of gomerulosa narrow at junction with fasciculata. Ink perfused.

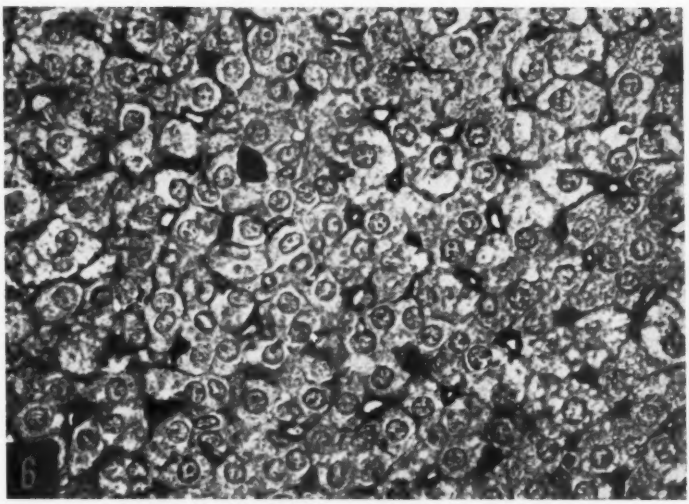

FIG. 6. Tangential section: every cell in fasciculata touched by at least one sinusoid. Ink perfused.

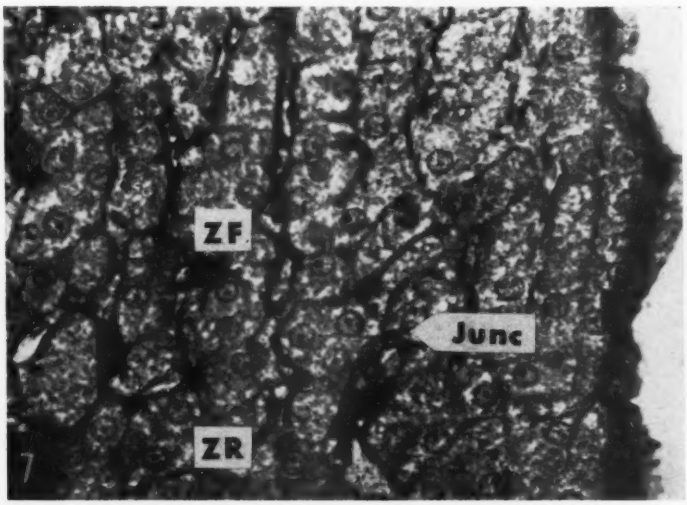

FIG. 7. Radial Section: small sinusoids of fasciculata increase in width at junction with reticularis. Ink perfused.

majority of the cells are touched by two vessels that have a relatively small diameter.

5. VESSELS OF THE ZONA RETICULARIS

As the sinusoids of the zona fasciculata pass into the zona reticularis they increase in width (Fig. 7). In the outer portion of the zone, these vessels

are approximately of the same diameter as those of the zona glomerulosa. Anastomoses are very frequent. As the sinusoids approach the medulla, they increase in width even more and finally join to form collecting tributaries of the suprarenal vein. The vessels of the zona reticularis do not run toward the medulla in a radial, parallel manner as do those of the zona fasciculata. Instead, their course is irregular; as they approach the medulla, they do so in an oblique direction.

6. ARTERIES TO THE CORTEX AND MEDULLA

A certain percentage of the blood supply to the cortex comes from cortical arterioles that originate in the subcapsular plexus. These vessels were first described in the adrenal of the dog by Flint (10) and in the mouse and rat by Gersh and Grollman (9). They penetrate the cortex, giving off small branches to the sinusoids in the various zones. Bennett and Kilham (8) in the cat, and Gersh and Grollman (9) and Lever (7) in the rat have described vascular loops arising in the subcapsular plexus that penetrate the cortex only to return to the subcapsular region. Branches from such loops and their distal segments supply the various zones. Such vessels are clearly revealed in this study. Although a certain small percentage of the blood going to the medulla passes into it from the sinusoids of the zona reticularis, the greater amount comes from medullary arteries (10) that pass through the cortex, without branching, and ramify in the medulla (Fig. 8).

FIG. 8. Radial section: medullary arteriole.

B. *Observations on the Stroma*

1. CAPSULE

a. *Collagen Fibers.* A tangential section through this region stained by the van Gieson method (Fig. 9) shows the fibers interlaced over the surface of the gland. A cross section of the capsule stained similarly (Fig. 10) reveals many dots, short lines, and an occasional long line. These are collagen fibers cut in cross, oblique, and longitudinal section, respectively. They are confined largely to the capsule. Exceptions to this are the fibers accompanying the larger vessels that project through the cortex to supply the medulla directly (Fig. 8) and an occasional fiber that projects into the

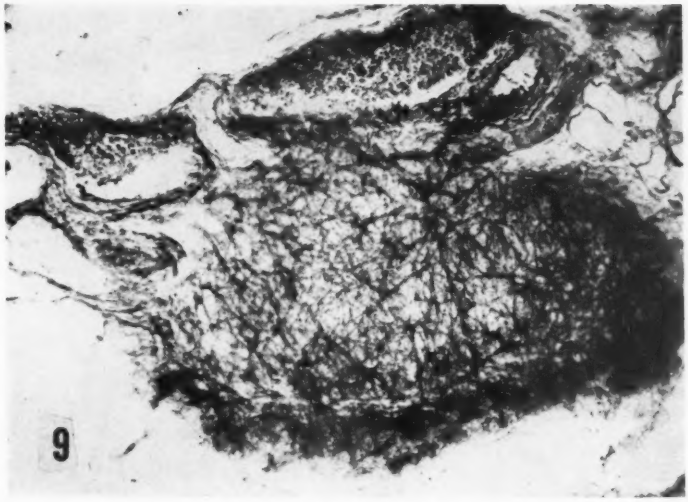

FIG. 9. Tangential section of capsule: collagen fibers interlace over surface of gland. Van Geison stain.

zona glomerulosa for a short distance. A gradual transition from broad, wavy, red fibers to thin, pink fibers occurs at the junction of the capsule and the outer cortex (Fig. 10).

b. *Argyrophil Fibers.* Silver impregnation of the stroma by the method of Gomori (Fig. 11) produces a pattern very similar to that produced by

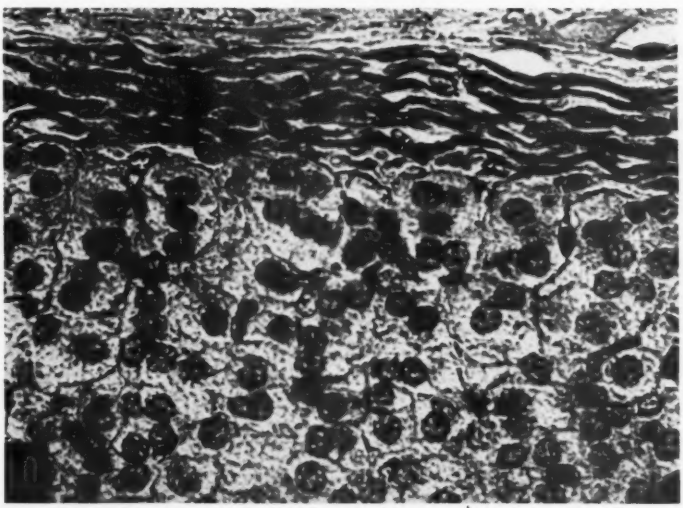

FIG. 10. Radial section of capsule: collagen fibers cut in cross, oblique and longitudinal section. Van Gieson stain.

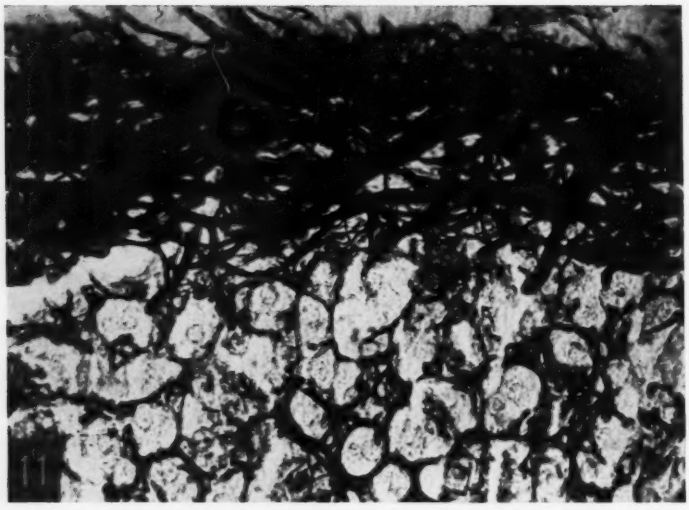

FIG. 11. Radial section: transition of thick, silver impregnated fibers of capsule to delicate fibers in glomerulosa. Individual cells of glomerulosa are supported by reticular fibers.

the van Gieson stain. There is a gradual transition from the thick fibers of the capsule to the delicate argyrophil fibers found at the junction of the capsule with the cortex.

2. ZONA GLOMERULOSA

a. *Collagen Fibers.* A few collagen fibers from the capsule penetrate into the zona glomerulosa. These fibers are closely associated with the larger vessels of the region. As the fibers penetrate deeper into the zona glomerulosa, they become thin and take a weaker stain. Collagen membranes as found in man (11, 12) (Fig. 18) separating large parenchymal masses from each other do not exist in the rat cortex.

b. *Argyrophil Fibers.* Silver impregnation by the method of Gomori shows that the argyrophil fibers of the cortex are continuous with those of the capsule (Fig. 11). Thick fibers from the capsule seem to be frayed and to be continuous with the thin fibers of the cortex. A close examination of the zona glomerulosa reveals small lines and dots around and between the individual cells (Fig. 11). By focusing the microscope up and down, it can be seen that these are reticular fibers cut in cross and tangential section. Occasionally a net of reticular fibers can be seen in the plane of the section. Argyrophil fibers surround the individual cells and support them; in this respect, the rat is different from all the other animals studied.

3. ZONA FASCICULATA

a. *Collagen Fibers.* The only van Gieson positive fibers that can be identified in this zone are those that contribute to the adventitia of the

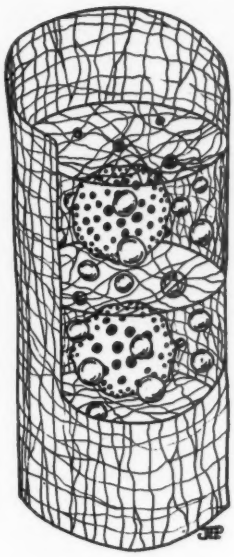

FIG. 12. Steriogram of reticular fibers surrounding cells of fasciculata.

larger blood vessels. This is especially noted in the case of the medullary arterioles.

b. *Argyrophil Fibers.* Fine reticular fibers surround the blood vessels and also penetrate between and surround the individual cells of this zone (Fig. 12). Thicker fibers around the larger vessels are continuous with the smaller fibers surrounding the parenchymal cells.

4. ZONA RETICULARIS

a. *Collagen Fibers.* In the outer part of this zone, the collagen fibers are again associated with only the larger blood vessels. Near the junction of the cortex with the medulla, however, these fibers often accumulate. Fuchsinophilic material and very tiny, van Gieson positive fibers can often be seen between degenerating cells (Fig. 13). In some areas, the collagen fibers form a definite boundary between the cortex and medulla and appear like an incomplete medullary capsule (Fig. 14). In other areas, no collagen fibers are associated with the junction. A few fibers running at oblique angles through the zona reticularis are occasionally observed; they are associated with some of the larger sinusoids.

b. *Argyrophil Fibers.* The large fibers which are closely associated with the large blood vessels branch and give rise to reticular nets that surround individual cells.

C. *Observations on the Parenchyma*

1. CAPSULE

At birth and at three weeks the capsule is very cellular, and these cells are morphologically similar to those of the zona glomerulosa. It is, there-

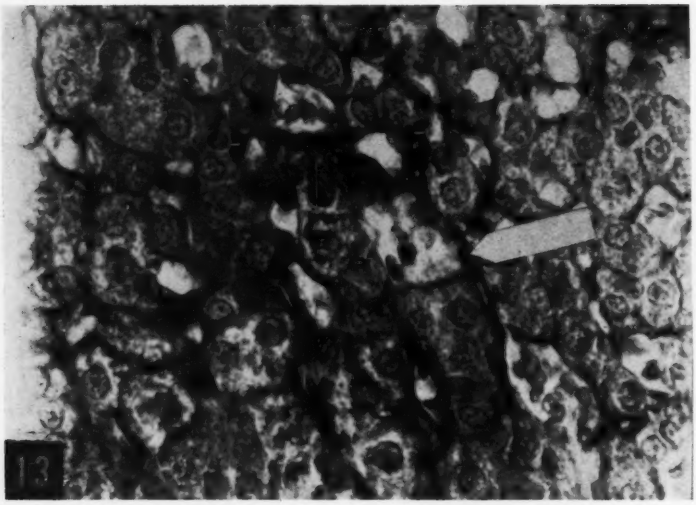

FIG. 13. Fuchsinophilic material and van Gieson positive fibers within and between cells of reticularis.

fore, difficult to determine exactly where the capsule ends and the glomerulosa begins. Jayne (13), on the basis of this same observation, suggested that these capsular cells give rise to those in the glomerulosa. Martinovitch (14) described two distinct layers of cells in the capsule, an outer layer composed of elongated cells with spindle-like nuclei and an inner layer of

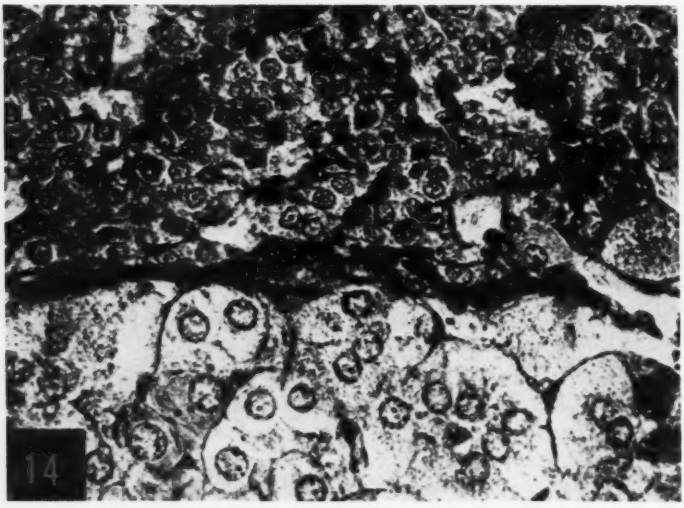

FIG. 14. "Medullary capsule." An accumulation of collagen fibers between reticularis and medulla.

"closely packed cells with small irregular or lobulated nuclei staining intensely with haematoxylin." Mitotic figures were occasionally found in the outer layer of the capsule in rapidly growing glands. They were never found in the inner layer. He supported the view of Greep and Deane (15) that the cells of the cortex give origin to all new cortical cells and suggested that the dividing cells in the outer layer of the capsule were indicative of capsular growth rather than cortical cell replacement. In the present study, the adrenals from animals 15 and 23 days old that had been injected previously with a dose of colchicine sufficient to arrest mitosis at the metaphase (16) show numerous mitotic figures in the zona glomerulosa and the outer part of the zona fasciculata. These figures are concentrated primarily just beneath the capsule, and others are noted at the junction of the zona glomerulosa and the fasciculata. No mitotic figures, cords, plates or large masses of cells are noted in the capsule.

2. ZONA GLOMERULOSA

Radial sections of cortices that have been perfused with ink in the region of the zona glomerulosa reveal a field of cells interrupted by blood vessels cut obliquely, longitudinally, and horizontally (Fig. 3). Tangential sections through the zona glomerulosa (Fig. 2) give the same impression, that is, a field of cells interrupted by blood vessels cut at all angles to the capsule. If the parenchyma of Figure 3 is interpreted as being composed of cords, then a tangential section through the same area should show a predominance of cross sections of these cords; however, Figure 2 does not show such cross sections. The fact that only longitudinal sections of the alleged cords can be seen suggests that these must be part of a continuum. This interpretation was confirmed by reconstructions.

3. ZONA FASCICULATA

Radial sections in the region of the zona fasciculata (Figs. 3 and 15) show a field of cells and parallel, longitudinal sections of blood vessels. The parenchyma may be interpreted as being composed of either cords of cells or a continuum. If it is composed of cords, then a tangential section through the same area should show round groups of cells separated from each other. Figure 6 is a tangential section through the zona fasciculata and shows a field of cells interrupted by cross sections of small blood vessels. No division of the cells by either membranous connective tissue, vascular sheaths, or intercellular spaces is evident. This again suggests that this zone is a continuum; verification was made by reconstruction (Fig. 16). In this respect, the zona fasciculata shows a structure identical to that in the horse (17) and it is thus radically different from the human zona fasciculata (11, 12).

A study of the reconstructions and serial sections shows that each cell in the zona glomerulosa and fasciculata is touched on at least one of its sur-

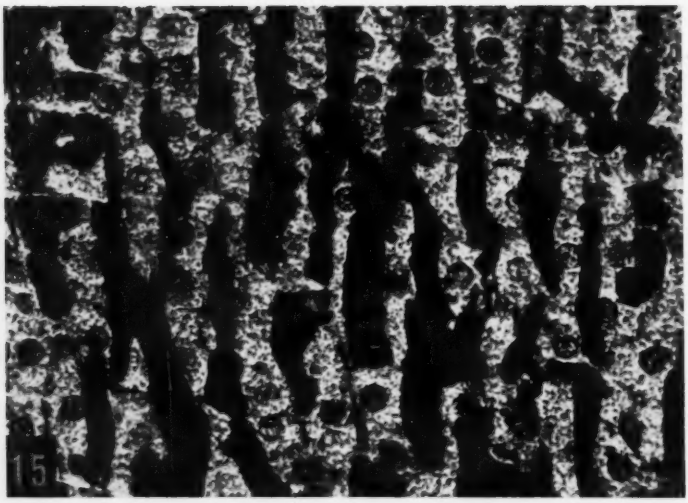

FIG. 15. Radial section of fasciculata. A field of cells and parallel, longitudinal sections of blood vessels. Ink injected under pressure.

faces by a sinusoid. In the zona glomerulosa, the vessels are of larger diameter than those in the middle zone and more of the surface of the individual cell is exposed to the sinusoid.

The parenchyma of this zone is a continuum, except for reticular fibers that form loose basketworks around the cells and the sinusoids that penetrate between them. Reconstructions fail to indicate any evidence for the "cord" concept in the zona glomerulosa or fasciculata of the rat.

4. ZONA RETICULARIS

Radial sections of the zona reticularis show that the thin sinusoids of the zona fascinculata have now increased in size and become tortuous in their

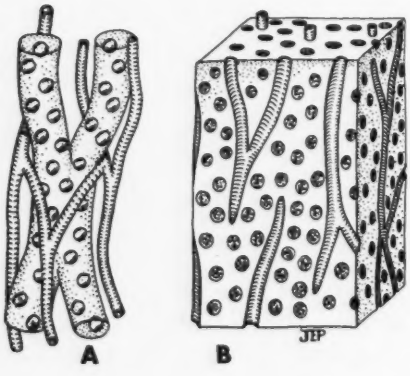

FIG. 16. Steriogram of zona fasciculata. *A*, old concept of cords of cells surrounded by vessels. *B*, reconstruction showing continuum of cells tunnelled by vessels.

course through the parenchyma as has been previously described. In radial section (Fig. 17), the cells of the zona reticularis form long, connected rows that, for the most part, are only a single cell in thickness. Between them are the large sinusoids. Reconstructions show that the parenchyma is composed of plates 1 or 2 cells thick, extensively tunneled by the sinusoids. Exceptions to this are the small groups of cells that can be found isolated from the rest of the parenchyma by the anastomoses of the sinusoids.

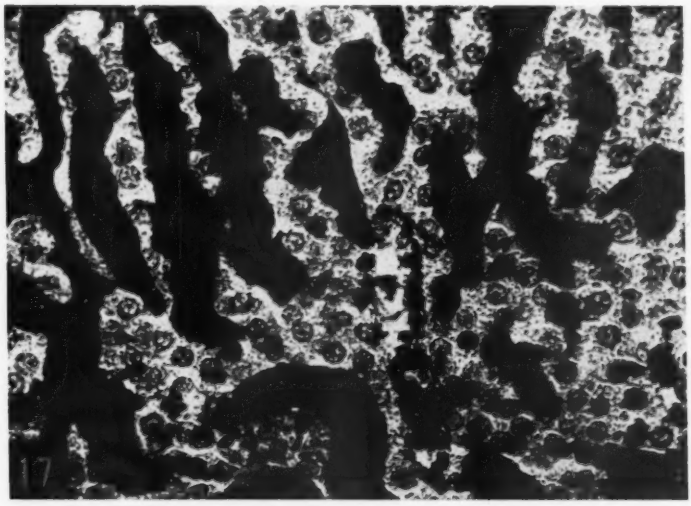

FIG. 17. Radial section of reticularis. A field of cells interrupted by longitudinal and oblique sections of large sinusoids. Ink injected under pressure.

Although degenerating cells can occasionally be found in all 3 zones, most of them occur in the zona reticularis. As in man (12) they are characterized by fuchsinophilic material that seems to accumulate in the cytoplasm and extracellular substance surrounding these cells (Fig. 13). The fuchsinophilic material is found especially in older animals.

DISCUSSION

In studies of the capsule of the dog adrenal, Flint (10) described an extensive arterial plexus, from which small arteries supplying the capsule branch. This capsular plexus must be unique for the dog, because it has not been found in the cat (8), mouse (9), rabbit (6), rat (9, 7), man (11, 12) nor by the present worker.

Gatz (18), in an unpublished report, fed rats silver nitrate in their drinking water and noted that the silver particles were phagocytized by the endothelial cells of the vessels in all 3 zones of the cortex. It appears, therefore, that these vessels are part of the reticulo-endothelial system.

On the basis of this finding they may be classified as sinusoids. Although the original definition of this term[4] is no longer tenable (4), it is usually customary to so refer to capillaries lined by potential phagocytes (by this term). Also, in spite of having shown that Minot's definition is incorrect, Elias (4) retains the term for the specialized capillaries in the liver.

Reconstructions have shown for the first time that every cell in the adrenal cortex of the rat contacts a sinusoid on at least one surface (Fig. 6). Therefore, the exchange of nutrients and metabolites between the intracellular and intravascular compartments is probably direct and not through other cells.

Although other workers (7) have noted the change in the diameter of the sinusoids from the zona fasciculata to the zona reticularis, any change of diameter from the zona glomerulosa to the zona fasciculata has previously been explained only on the basis of lipoid distribution (8). Bennett and Kilham suggested that the vessels of the outer portion of the zona fasciculata are compressed by the abundance of lipoids. Lever (7) supported their findings by showing that the greatest amount of lipoid was in the zona glomerulosa and the outer part of the zona fasciculata. Our lipoid preparations confirm this distribution. However, if they were correct in their assumption that the vessels are compressed by the surrounding lipoid-laden cells, then one should expect the cross sections of the vessels in this region to have a crenated or flattened appearance. Figure 6 shows that this is not the case. Therefore, the size of these vessels is probably not governed by the amount of lipoid impinging on their surfaces, but is an inherent characteristic of the zone.

The difference in diameter and direction of the vessels as they cross from one zone to another suggests a possible means of identification of the individual zones without reference to their cellular morphology. This may be a useful tool with which to identify zones whose cytology has been altered by various experimental procedures.

Elias and Pauly (11, 12), in a study of the human adrenal cortex, found that the sinusoids of the zona fasciculata are dilated to such an extent that their endothelial walls touch one another. Consequently, vascular sheaths surround portions of the parenchyma, dividing it into cords (Fig. 18). This is not the case in the rat, where one finds vessels of much smaller diameter and parenchyma not divided into cords. The human zona fasciculata is much more vascular than the rat's, which resembles that of the horse.

Flint (10) maintained that the individual connective tissue fibers do not

[4] "A sinusoid may be defined as a subdivision of a vessel produced by intercrescence between its endothelium and the parenchyma of an adjacent organ. The proliferating tubules or trabeculae of an organ encounter a large vessel and invade its lumen, pushing the endothelium before them" (19).

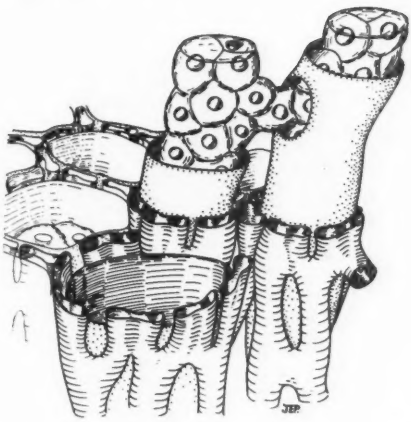

FIG. 18. Stereogram of human adrenal fasciculata. Cords of cells surrounded by collagenous sheaths between which lie enlarged blood sinusoids so numerous that their endothelial walls touch each other.

run between the cells in the zona glomerulosa of the dog. Lever (7) noted the honey-comb pattern of reticulum in the zona glomerulosa of the rat and said that the fibers surround small clusters of the parenchymal cells. Elias and Pauly (11, 12) found relatively large masses of zona glomerulosa

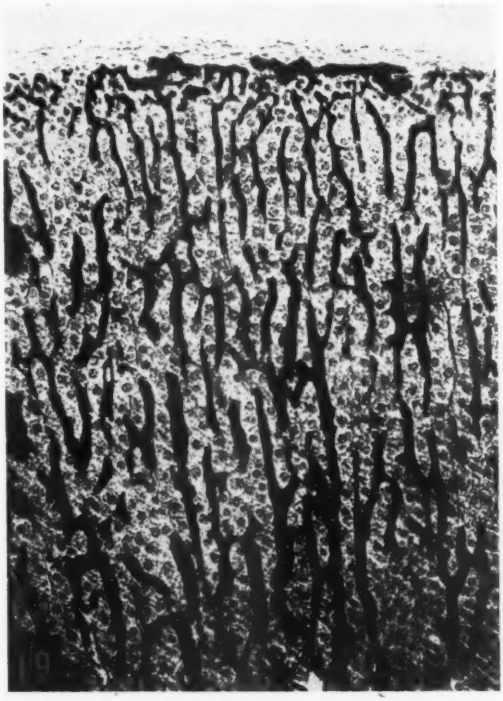

FIG. 19. Radial section of rat adrenal cortex showing "cords."

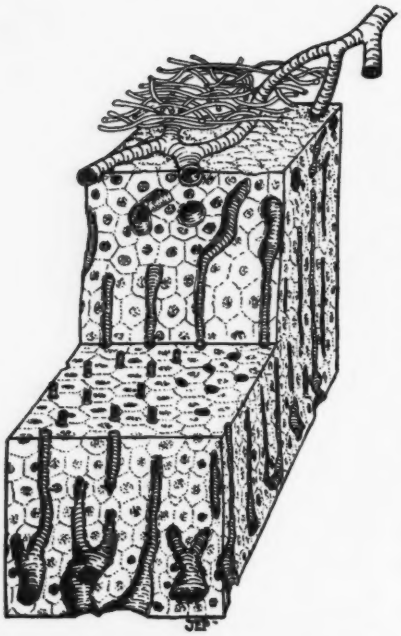

FIG. 20. Stereogram of rat adrenal cortex. Parenchyma is a tunnelled continuum.

separated by collagen membranes in the human adrenal cortex. In the rat, these membranous divisions do not exist. However, for the first time argyrophil fibers have been demonstrated penetrating between the individual cells in this zone and supporting them in a reticular network. Although this network is not so dense as that found in the zona fasciculata or reticularis of the rat and other species (20), careful observation shows that it does exist.

The rat adrenal cortex, like that of many other species, has always been considered to consist of cords (21) (Fig. 16). The reason for this is that a radial section through the center of the gland (Fig. 19), cuts the parallel vessels of the zona fasciculata longitudinally, suggesting that the long rows of cells between the vessels are longitudinal sections of cords. However, tangential sections through this zone (Fig. 6) show that the cords do not exist.

Reconstructions have shown that the parenchyma of all zones is a tunnelled continuum (Fig. 20). In the zona reticularis, the dilated sinusoids occasionally do divide the parenchyma into short plates or cords, thus making an exception to the rule.

Arnold (1) said that the columnar structure of the human zona fasciculata is caused by the long axis of the connective tissue pillars between which lies the reticulum with the parenchymal bodies. Elias and Pauly (11, 12) showed that these columns of cells are surrounded by a collagenous sheath, between which lies enlarged blood sinusoids so numerous that their endothelial walls touch each other; thus, two parenchymal cords are separated from each other by two membranes and at least one sinusoid (Fig. 18). In the case of the rat, the sinusoids have a small diameter and are not so numerous as they are in the human; furthermore, membranous connective tissue does not exist in this zone.

A replacement of adreno-cortical cells by morphogenesis of indifferent cells of the capsule and end-to-end junction of these newly formed cords with those of the cortex was first suggested by Zwemer, Wotton, and Norkus (22) in the cat, monkey, man and unidentified rodents. Gruenwald and Konikov (20) added the guinea pig, rabbit, dog, pig and horse to the

list and described the apposition of a complete new layer from the capsule to the cortex as a second mechanism of replacement. However, they were unable to find any definite indication of apposition from the capsule of the rat in the sections they studied. Our observations indicate that the reason for this is that the laboratory rat under conditions of normal growth and cell replacement does not have a capsular blastema. The controversy that has arisen over the blastema theory is a result of different investigators studying different species. Since these many species are morphologically quite dissimilar, there is reason to suspect that their mechanisms of growth and cell replacement are quite different. Although other workers have shown that an intracapsular blastema exists in many species, the fact that we are unable to find it in the rat indicates to us that the controversy for and against the blastema theory may be resolved as a mere species difference.

SUMMARY

1. *Vasculature:* From a study of serial sections of ink perfused glands, large sinusoids arising from the subcapsular plexus were found to penetrate into the glomerulosa and course through the parenchyma at all angles to the capsule. At the glomerulosa-fasciculata junction, they become narrow and course through the fasciculata centripetally. Upon entering the reticularis, they dilate and become tortuous; approach the medulla at various angles and group together to form the primary radicles of the adrenal vein. The differences in diameter and direction of the vessels as they cross from one zone to another suggest a possible means of identification of the individual zones without reference to their cellular morphology. This may be a useful tool with which to identify zones in which the cytology has been altered by various experimental procedures. Reconstructions have shown for the first time that every cell in the 3 zones contacts a blood vessel on at least 1 surface.

2. *Stroma:* Silver impregnations and staining by van Gieson, trichrome and H and E technics demonstrate the capsule to be composed of collagen and reticular fibers. Except for occasional collagen fibers projecting into the cortex for short distances and those accompanying larger vessels, these fibers are confined to the capsule. Reticular fibers support the individual cells in all 3 zones, contrary to prior reports. The greatest concentration of reticulum occurs in the reticularis, the least in the glomerulosa.

3. *Parenchyma:* Reconstructions of serial sections show that the parenchyma of the glomerulosa and fasciculata is a tunnelled continuum and not cords of cells. The reticularis is also a continuum except for occasional small groups of cells separated by dilated vessels from the main parenchymal mass. Short plates and cords make up this zone, and degenerating cells are common.

4. Colchicine studies on the rat adrenal show that the greatest concen-

tration of mitotic figures is in the area just under the capsule. Although other workers have shown that an intracapsular blastema exists in other species, the fact that no mitotic figures, cords, plates or large masses of cells were found in the rat capsule indicates that the controversy for and against the blastema theory may be resolved as a mere species difference.

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IN VIVO EFFECTS OF GLUCAGON ON HEPATIC GLYCOGEN, PHOSPHORYLASE AND GLUCOSE-6-PHOSPHATASE[1]

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RECENT studies in our laboratory on rat liver slices have revealed a critical time relationship between the addition of glucagon to the medium and the observed levels of phosphorylase activity. This study in dogs was therefore undertaken to determine *in vivo* the degree and duration of alterations in this enzyme and the secondary changes in hepatic glycogen content. Also measured were hepatic glucose-6-phosphatase activity, blood sugar and lactate, and serum sodium, potassium and inorganic phosphorus.

METHODS

Mongrel dogs of both sexes weighing between 10 and 13.5 kg. were last fed 12 hours before the procedure. After light intravenous sodium pentobarbital anesthesia, hepatic exposure was achieved by either a midline or sub-costal incision with maximal hemostasis. Liver biopsies averaged 1½ grams, and the V-shaped wound in the lower edge of the lobe rapidly closed by suture. These were always several centimeters apart or randomly chosen from different lobes. Polyethylene catheters were placed in the femoral veins for infusions and blood sampling. Warm saline pads covered the abdominal wound between exposures. Each animal was sacrificed at the end of the experiment.

Amorphous glucagon,[4] with 50% activity compared to the crystalline substance, was used in all experiments. In the single injection procedures, 200 micrograms were dissolved in 1 ml. glycylglycine buffer, pH 8.7, and given intravenously. In the infusion experiments 3 ml. of this solution were diluted in 200 ml. isotonic saline and delivered at a rate of 2.5 micrograms glucagon per minute. Control animals received the buffer alone or dextrose in water.

Biopsies were immediately cooled in iced saline and analyzed. Whole blood was added directly to water for Somogyi-Nelson glucose determination (1, 2) and to 10% trichloracetic acid (TCA) for lactate analysis (3). Serum was removed from blood and aliquots analyzed for inorganic phosphorus (4) and for sodium and potassium by an internal standard flame photometer.

Each biopsy was divided into two ½-gram pieces, trimmed and weighed; one piece was digested in 30% KOH for total glycogen analysis and the other homogenized in

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[4] Obtained through the courtesy of Dr. W. R. Kirtley and Dr. M. Root of the Eli Lilly Laboratories, Indianapolis 6, Indiana.

6 ml. cold water. Aliquots of the latter were taken for enzyme determinations and an equivolume of 10% TCA added to the remainder. This supernatant fluid and the KOH digest were each mixed with 1.2 volumes 95% ethanol and the precipitated glycogens resuspended in water and directly measured with modified anthrone reagent (5).

Phosphorylase was determined by incubation of 0.1 ml. homogenate in a mixture of 0.2 ml. 0.154 molar citrate buffer, pH 5.9, and 0.3 ml. 0.154 molar sodium fluoride with 0.05 ml. of 0.2 molar glucose-l-phosphate as substrate for 30 minutes at 30° Centigrade. One ml. of 10% TCA terminated the reaction and inorganic phosphorous was measured (4). Glucose-6-phosphate was similarly determined but in citrate pH 6.4 with glucose-6-phosphate as substrate and in the absence of fluoride. After subtraction of tissue and reagent blanks, values were expressed as micromoles inorganic phosphate released from the substrate per gram of wet liver per 30 minutes.

RESULTS

In Figure 1 are graphed the results of four dogs given a single intravenous injection of 200 micrograms of glucagon. Blood glucose rises rapidly, reaches a maximum at thirty minutes and falls toward normal by two hours. Liver glycogen decreases in a similar fashion; the decrement however is from the TCA-soluble fraction. Phosphorylase activity more than doubles in fifteen minutes, falls below the control value in one hour and returns to slightly above the control value by two hours. In spite of the low level of phosphorylase activity one hour after the injection, glycogen continues to be broken down but at a slower rate compared to the initial 30 minutes. Liver glucose-6-phosphatase is unchanged.

Table 1 tabulates the serum electrolyte changes from two typical animals given the single injection of glucagon. Similar decreases in serum potassium and inorganic phosphorus were observed in all dogs. The slight fall in serum sodium was frequently but not uniformly seen.

More marked changes are observed when dogs are given a four-hour infusion of 2.5 micrograms of glucagon per minute. A typical animal is charted in Figure 2. Blood glucose peaks at one hour and falls as hepatic glycogen becomes depleted. As in the single injection experiments, the TCA-soluble fraction of glycogen is mobilized. The small additional fraction which is recovered by KOH digestion remains approximately stable. Phosphorylase remains elevated throughout the infusion, the slight fall possibly being due to inadequate glycogen in the enzyme assay mixture. Glucose-6-phosphatase activity increases gradually to about $1\frac{1}{2}$ times the initial value.

In the four-hour infusion experiments, serum phosphate and potassium showed decreases similar in degree and duration to the acute injection procedures. Blood lactate was measured in two experimental animals and it remained stable at 1.6 and 1.0 micromoles/ml. during the infusion, but rose slightly during the fourth to sixth hours in both the experimental and control animals.

The glucose infusion administered to the control animals was calculated to equal the amount and rate of carbohydrate released from the liver from

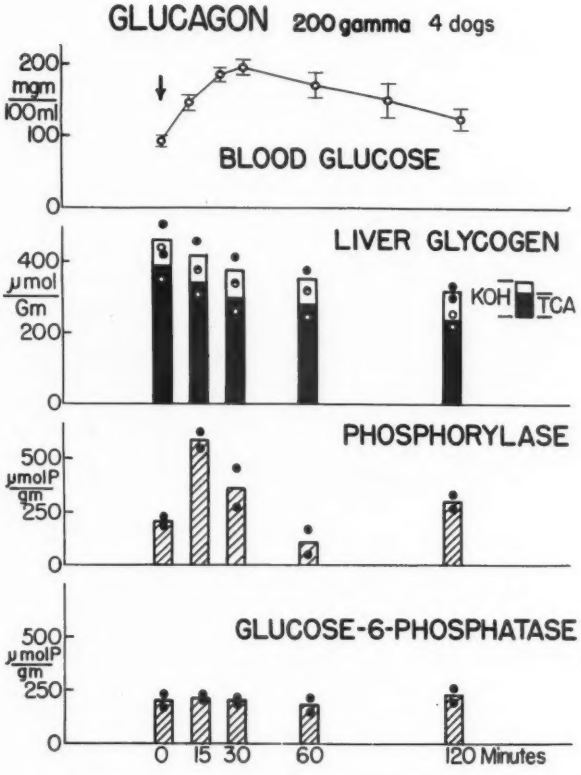

FIG. 1. Mean values and standard deviations from four dogs receiving a single injection of 200 micrograms glucagon at 0 minutes. The first set of data on each line represents the control or pre-glucagon values.

TABLE 1. SERUM VALUES FROM TWO DOGS RECEIVING A SINGLE INJECTION OF 200 MICROGRAMS GLUCAGON

Dog	Weight	Sex		Control	Minutes after glucagon				
					10	20	30	60	120
7	11 kilo	M	serum inorg. phosphorous mg %	6.1	5.6	4.4	4.5	5.0	6.4
			serum sodium meq/L	159	159	157	153	156	156
			serum potassium meq/L	4.4	4.1	3.7	3.7	4.1	4.5
8	10 kilo	M	serum inorg. phosphorous mg %	5.5	4.8	4.2	4.3	4.8	5.4
			serum sodium	160	156	153	153	151	151
			serum potassium	5.9	5.8	5.0	5.0	5.4	5.6

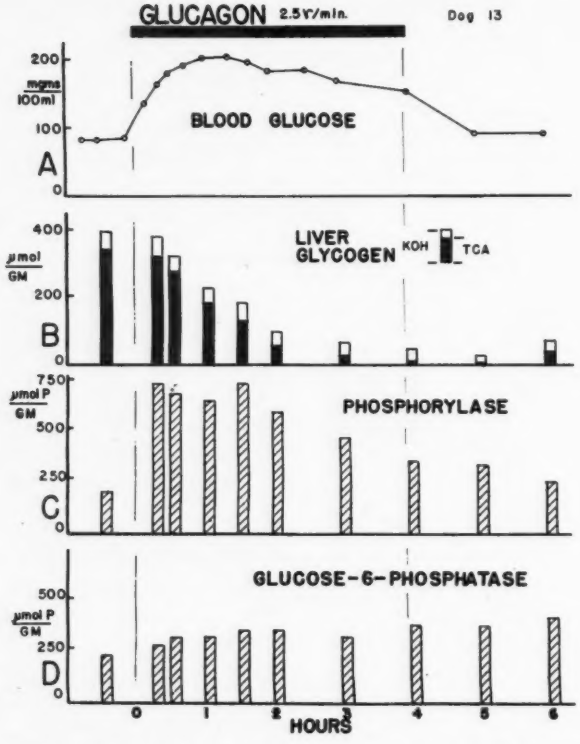

FIG. 2. A representative animal receiving a continuous infusion of glucagon, 2.5 micrograms per minute, starting at 0 hours and terminating at the fourth hour.

both the single-glucagon injected or the four-hour-glucagon-infused animals. In every case, blood glucose curves, and serum phosphorus and potassium decreases were of the same magnitude whether the carbohydrate was internally mobilized by glucagon or externally administered by means of a glucose infusion.

DISCUSSION

Increased phosphorylase activity has been associated with accelerated glycogenolysis (6, 7). The mechanism whereby this enzyme is able to alter the equilibrium distribution of its substrates is unknown. The single injection experiments show marked increases in activity of this enzyme associated with rapid glycogenolysis. However, at one hour (Figure 1) glycogenolysis continues in spite of a marked rebound of phosphorylase to below control levels.

The dogs receiving a four-hour infusion of glucagon contribute 3 grams glucose/kilogram body weight to the blood stream as calculated from liver weight and changes in glycogen content. Control dogs receiving this

amount of glucose over the same period of time as a continuous infusion show similar serum potassium and inorganic phosphate decreases but no alterations in hepatic enzyme content.

The liver of the dogs receiving the sustained glucagon infusion became perceptibly smaller during the experiment. Fenn (8) has calculated that for each gram of glycogen about 2 ml. of water accumulate in liver tissue and are lost when the glycogen is mobilized. Thus, a ten kilogram dog would lose 90 grams of liver weight with a loss of 30 grams of glycogen. This is approximately $\frac{1}{4}$ of the liver weight of a fed animal. All enzymes would therefore be expected to rise proportionately when expressed per gram of wet liver. Thus, the apparent elevation in glucose-6-phosphatase activity may be interpreted as concentration. This finding agrees with Ashmore, Hastings and Nesbett (9) who described the increased level of glucose-6-phosphatase per gram of liver in the fasted rat to be a result of a reduction in the over-all size of the organ.

The TCA-soluble fraction of glycogen was depleted during glucagon administration; the protein-precipitable moiety remained stable. Bloom *et al.* (10) have shown this latter fraction to be similarly stable after epinephrine or fasting.

SUMMARY

Glucagon given in a single injection to anesthetized dogs rapidly elevates liver phosphorylase activity resulting in a decrease in hepatic trichloracetic acid (TCA) soluble glycogen. A four-hour infusion of glucagon almost completely depletes hepatic TCA soluble glycogen and due to a diminished liver size, glucose-6-phosphatase activity rises when expressed per gram liver weight. Infusion of an amount of glucose expected to equal the amount of glycogen mobilized from the liver by glucagon results in similar serum potassium and inorganic phosphorus decreases and also in a similar glucose tolerance curve.

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EFFECT OF ESTROGEN AND ANDROGEN UPON UPTAKE OF GLYCINE-C^{14} BY RAT UTERI *IN VITRO:* LACK OF CONCENTRATION DEPENDENCE[1]

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IN A recent report (1) dealing with the effect of testosterone propionate (T.P.) upon incorporation of labeled glycine into mouse kidney slices, data were presented showing that the effect of T.P. appeared to depend upon the concentration of glycine in the incubation medium. Thus, treatment of female A/Jax mice with 1 mg. T.P./day for 2 days resulted in an apparent increase of 35–70% in incorporation rate when the concentration of glycine in the medium was 1.0 mM or lower; when the concentration of the latter was increased to 5 mM or more, the difference between incorporation rates for control and treated animals was less than 20%. These facts were interpreted as indicating that T.P. acts, in part at least, by facilitating the intracellular accumulation of glycine.

The incorporation of radio-glycine into proteins of surviving segments of rat uteri has been shown to be substantially increased by the administration of testosterone (2) as well as estradiol (2, 3). Simultaneous administration of both hormones effected an increase greater than either alone (2), leading to the suggestion that different mechanisms were involved in each case.

A study of the concentration dependence of the effect of these two hormones upon glycine uptake by rat uteri was undertaken, therefore, to determine, first, the extent to which the effect of testosterone upon the rat uterus paralleled its action upon the mouse kidney; and second, to ascertain if the actions of estrogen and androgen could be differentiated in terms of concentration dependence.

EXPERIMENTAL

Female white rats, 60 days old were ovariectomized and allowed to rest four weeks or longer. Estradiol-17β and testosterone propionate[2] were injected as solutions in

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[2] Neohombreol; Organon, Inc.

sesame oil containing 100 μg./ml. and 10 mg./ml., respectively. One-tenth ml. of solution was injected daily for two days. Twenty-four hours after the second injection, the animals were killed by a blow on the head, the uterus was removed, the two horns separated at the cervix, and the segments slit longitudinally. Fifty to 100 mg. of tissue were placed in a flask with 2 ml. of medium and incubated two hours at 38.5°. Details of the composition of the medium and preparation of protein samples for counting have been given previously (1).

RESULTS

The data include the results of experiments with four groups of rats—untreated controls, an estrogen-treated group, a T.P.-treated group and a fourth set which received both hormones.[3] For each group, experiments were run at four concentrations of glycine—0.25, 1.0, 3.9 and 8.2 mM.

Figure 1 shows how the incorporation rate depends upon the ambient glycine concentration for the control and estrogen-treated series. In Figure 2, the increase in incorporation rate for each group, compared to untreated controls, is plotted against the glycine concentration.

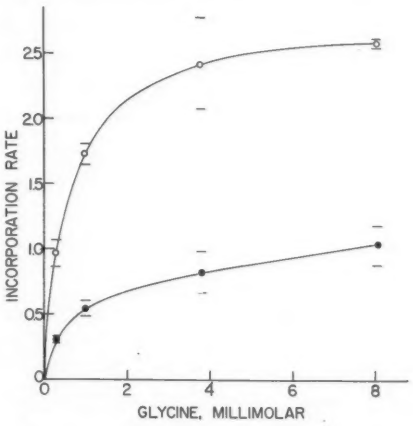

FIG. 1. Effect of total concentration of glycine upon incorporation into uterine proteins of ovariectomized (—●—) and ovariectomized, estrogen-treated (—○—) rats. Ordinate: incorporation rate in micromoles of glycine carbon per gram of protein per hour. Horizontal lines bracket two standard errors about the mean.

It first of all may be observed that estrogen and testosterone, under the conditions of these experiments, increased incorporation into proteins of the uterus by 230% and 100%, respectively when the glycine concentration was 1.0 mM. These increases are of the same general magnitude as observed previously by Mueller (2), using somewhat different injection schedules. Although Mueller found that the simultaneous administration of estradiol and testosterone 6 hours before sacrifice resulted in incorporation rates greater than with either steriod alone, our data indicate a slight inhibition of the estrogen effect by testosterone (Fig. 2). It seems likely that differences in experimental design may account for this discrepancy.

[3] A total of 72 rats were used in the experiments reported here; each point of figures 1 and 2 represents the mean of four to eight determinations. The maximum standard error for any point was less than 20% of the mean.

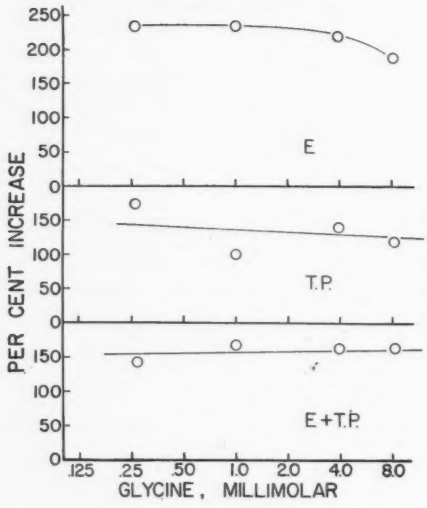

FIG. 2. Concentration dependence of the actions of estradiol (E), testosterone propionate (T.P.) and their combination (E+T.P.) upon uptake of glycine by rat uterine segments. Ordinate: per cent increase in incorporation rate compared to untreated controls.

Figure 2 indicates that stimulation of incorporation into rat uterine protein by both estrogen and T.P. is essentially independent of the glycine concentration in the surrounding fluid. The primary sites of action of these two hormones would therefore appear to be at a metabolic locus one or more steps removed from that which controls intracellular accumulation of substrate. Whether the mechanisms of action of the two steriods bear closer similarities must be decided by further experiment; it would seem, however, that the action of testosterone propionate upon the rat uterus differs qualitatively, as well as quantitatively, from its action upon the mouse kidney.

SUMMARY

The administration of 20γ estradiol or 2 mg. testosterone propionate to ovariectomized rats during two days stimulated incorporation of labeled glycine into uterine protein *in vitro* by 230% and 100%, respestively. Upon simultaneous injection of both hormones, an increase of 170% was observed. These effects were independent of glycine concentration over the range 0.27 to 8.2 mM.

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EFFECT OF TESTOSTERONE PROPIONATE ON MYOSIN, COLLAGEN AND OTHER PROTEIN FRACTIONS IN STRIATED MUSCLE OF GONADECTOMIZED RATS

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GONADECTOMY decreased the rate of formation of collagen and alkali soluble stroma in the rectus femoris muscle of the male guinea pig but had no effect on the formation of myosin, "water soluble" (sarcoplasmic) proteins and non-protein nitrogen (1). However, in the temporal muscle the formation of all fractions was retarded markedly by gonadectomy. The rates of formation of all fractions in both muscles were normal in gonadectomized male guinea pigs treated with testosterone propionate.

The present experiments were conducted in order to determine if a similar effect on muscle protein fractions occurs in the thigh muscle of the male rat following gonadectomy.

METHODS AND MATERIALS

Male Sprague-Dawley rats from the National Institutes of Health animal colony were fed *ad libitum* diet 4164 (2) and maintained in individual cages. Of the rats received on a given day, one-third were kept as controls and the others were gonadectomized at 20 to 23 days of age. Beginning on the third day after the operation, one-half of the operated animals were injected subcutaneously each day with 0.1 mg. of testosterone propionate (in sesame oil) per 100 gm. body weight for 56 days. When rats were autopsied on a given day, representative animals from each group were taken.

While under chloroform anesthesia, the rats were exsanguinated through a large cardiac incision and the right thigh muscle was then quickly removed, weighed, and placed on solid carbon dioxide. The method of isolating the various fractions was the same as that used in the guinea pig and has been described earlier (1). The weight of the empty seminal vesicles was determined and used as an index of completeness of gonadectomy.

RESULTS

There was no effect of gonadectomy and of testosterone in gonadectomized rats on body weight, nose-tail length, and weight of the thigh muscle (Table 1). Gonadectomy resulted in a profound atrophy of the seminal vesicles, which could be completely prevented by testosterone.

There was no effect of gonadectomy and of testosterone in gonadec-

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TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE ON BODY WEIGHT AND LENGTH AND ON WEIGHT OF THE THIGH MUSCLE IN GONADECTOMIZED RATS[1]

Group	Age days	No. of rats	Body weight (gm.)		Nose-tail length (cm.)	Thigh muscle[2]		Seminal vesicles[3] (mg.)
			Final	Gain		Gm.	Gm./100 gm. body weight	
Normal	23	6	70.3±2.3	—	25.0±0.4	2.05±0.05	3.13±0.06	12.5± 0.3
Normal	80	7	279.6±6.2	200.7±4.8	40.4±0.4	11.41±0.22	4.28±0.03	384.3±22.8
Gonadectomized[4]	80	7	277.0±8.9	204.8±6.6	40.6±0.4	11.91±0.39	4.49±0.05	8.9± 0.9
Gonadectomized-treated[4]	80	8	279.9±4.7	209.4±4.4	39.9±0.5	11.67±0.27	4.40±0.04	440.1±37.4

[1] Mean ± standard error (Standard error = Range/number of animals (Mantel)).
[2] Consists of quadriceps, hamstring, adductor, and gluteal muscles and related tendons, fascia, nerves, blood vessels and fat.
[3] Empty weight.
[4] Gonadectomized at 21–23 days of age; testosterone propionate, 0.1 mg./100 gm. body weight daily, was begun on the third postoperative day and continued for 56 days.

tomized rats on the proportions and amounts of the different protein fractions in the thigh muscle (Table 2). Both the ratios of actomyosin to myosin and of myosin to collagen were normal in treated and untreated operated animals. There was a small but statistically significant difference between the young and old normal rats in the proportion of myosin to collagen in the muscle.

DISCUSSION

Gonadectomy and testosterone administration had no effect on the formation of the various protein fractions or on the weight gain of the thigh muscle in the growing rat. An earlier observation (3) that a small reduction in the collagen content of this muscle occurred after gonadectomy was not confirmed. Korner (4) found no effect of methylandrostenediol in adult female rats on the protein content of myofibrillar, sarcoplasmic and collagen fractions of several different striated muscles. The weight increments in these muscles were less than 10%. Stewart (5) observed in normal male adult rats implanted with testosterone pellets that the 39% increase in weight of the levator ani muscle was associated with a comparable percentage increase in the contractile, sarcoplasmic and connective tissue protein fractions.

The effect of androgens in rats, either normal or castrated, on body weight gain and on the growth of striated muscles, with the exception of

TABLE 2. EFFECT OF TESTOSTERONE PROPIONATE ON THE PROPORTION OF VARIOUS NITROGEN FRACTIONS IN THE THIGH MUSCLE OF GONADECTOMIZED MALE RATS[1]

Group	Age days	No. of rats	Thigh muscle[2]							
			Protein (N×6.25) % of wet weight	Nitrogen in percent of total nitrogen					Myosin/ Collagen	Actomyosin/ Myosin[3]
				Myosin[3]	"Water-soluble" protein	Non-protein nitrogen	Alkali-soluble stroma	Collagen		
Normal	23	6	17.70±0.19	38.29±0.11	36.85±1.17	17.32±1.28	4.57±0.39	2.76±0.15	14.1±1.0	77.4±1.5
Normal	80	7	19.90± .036	40.45±1.21	35.43±1.13	17.38±0.91	2.74±1.06	3.93±0.14	10.3±0.6	82.8±2.2
Gonadectomized	80	7	20.24±0.36	41.96±1.06	34.49±0.59	17.64±0.69	2.24±0.40	3.66±0.19	11.7±1.0	77.1±2.0
Gonadectomized-treated	80	8	19.59±0.22	41.93±0.68	33.70±0.49	18.00±0.23	2.35±0.42	3.99±0.11	10.6±0.4	79.8±1.1

[1] Mean ± Standard Error.
[2] Consists of quadriceps, hamstring, adductor and gluteal muscles and related tendons, fascia, nerves, blood vessels and fat.
[3] The myosin fraction is a mixture of actomyosin (myosin B) and myosin A. (Szent-Györgyi).

the perineal musculature (muscles of the penis and the levator ani), is always small, seldom more than 10%, and often statistically insignificant (3, 6, 7, 8, 9, 10, 11). It is possible that these small increments may be the effect of an increased food intake, which has been observed in treated animals (12).

The weight increase of the levator ani muscle in the rat is now extensively used as an index of the myotrophic activity of androgens (8). The use of the term "myotrophic activity" implies that the response of this muscle is representative of that of the other muscles in the body. Korner and Young (10) observed differences in the weight gain of various striated muscles (levator ani not studied) when methylandrostenediol was injected in the rat, but the magnitude of these responses was less than 15%. In castrated and in hypophysectomized rats, testosterone injections produced more than a 500% increase in the weight of the levator ani muscle with no effect on the weight of the thigh muscle or heart (3). The levator ani muscle in the rat is not comparable to that in man; this muscle in the rat appears to be a sphincter of the rectum, for it encircles the rectum, and is attached to the bulbocavernosus, one of the muscles of the penis (8). The response of the levator ani to androgens then should not be called a myotrophic effect since the term myotrophic implies that the hormone affects all muscles in a similar manner.

In the guinea pig, there are several striated muscles that have marked sensitivity to testosterone (13, 14). Kochakian *et al.* (14), in a study of 49 different muscles, observed that the androgen-sensitive muscles, except for the retractor penis muscle, were located in the head and neck region. There was only a 10% reduction in "total" muscle mass at 12 weeks and 20% at 27 weeks after castration. Scow and Hagan (1) have demonstrated that gonadectomy affected all protein fractions in the temporal muscle of the guinea pig, whereas only two fractions in the rectus femoris were affected. The fierce fighting with teeth which occurs between the males, especially among females in estrous,[1] would suggest that the hypertrophy of the head and neck muscles is a very important male sexual character in the guinea pig, such as the growth of the antlers in the deer (15). Because of the special role that the head and neck muscles play in the sexual behavior of the male guinea pig, the weight responses of these muscles to the male hormone should also be considered as an androgenic effect. It is possible that in other species testosterone stimulates the growth of certain muscles, but only those important in the sexual activity of the male.

SUMMARY

The effect of testosterone propionate on the thigh muscle in male rats gonadectomized at weaning was studied by determining the amounts of

[1] Personal communication from Mr. Samuel M. Poiley of the Animal Production Section of the National Institutes of Health.

the following nitrogen fractions in muscle: Myosin (A and B), actomyosin (myosin B), "water-soluble" (sarcoplasmic) proteins, non-protein nitrogen, alkali-soluble stroma and collagen.

Gonadectomy and testosterone administration for 56 days had no effect on the growth of the thigh muscle and the amounts of the various nitrogen fraction in this muscle.

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STUDIES ON THE METABOLISM OF GLUCAGON-I^{131} IN RATS[1]

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THE lack of pure preparations of glucagon has impeded studies of its metabolism in the past. As the result of recent investigations, glucagon has been isolated in crystalline form (1), and its structure has been clarified (2). The ability to radioiodinate glucagon without loss of biological activity has facilitated the study of the metabolism of the compound. This paper describes experiments designed to elucidate the distribution and degradation of glucagon-I^{131} and its excretion in urine and feces following administration of the material into the caudal vein of rats. The distribution and degradation of glucagon-I^{131} following its injection into the portal vein have also been compared with its fate after injection into the inferior vena cava.

MATERIALS AND METHODS

Crystalline glucagon[4] labeled with I^{131} [5] was used in all experiments. Specific activity ranged from 0.41 to 1.1 mc./mg. No loss of biological activity, from that present in the starting material, could be detected by bioassay based on the hyperglycemic response in dogs,[5] or by *in vitro* assay, using the liver slice method of Sutherland and de Duve (3).[6]

Male Sprague-Dawley rats, weighing 190–250 gm. were used in all studies. The animals were fasted, but had free access to water, for 17–18 hours prior to use. Sodium pentobarbital anesthesia was used in all experiments. The intravenous injection of 0.5 μg. of glucagon produced significant hyperglycemia in rats prepared in this fashion.

Glucagon-I^{131}, in the quantities described later, was administered in 0.5 ml. of 0.9% saline by tail vein, inferior vena cava, or portal vein. Those rats which were operated upon received the hormone 2 minutes after completion of the procedure. The rats were sacrificed by aortic exsanguination at 5, 15, 30, or 60 minutes, and the tissues carefully removed. Radioactivity was assayed in a well-type gamma detector after precipitation

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[2] Trainee, National Institute of Arthritis and Metabolic Diseases.

[3] Public Health Research Fellow, National Institute of Arthritis and Metabolic Diseases.

[4] Kindly supplied by the Eli Lilly Co., Indianapolis, Indiana.

[5] Kindly performed by Dr. A. Staub, Veterans Administration Hospital, Denver, Colorado.

[6] Kindly performed by Dr. J. M. Tyberghein, University of Washington.

with trichloroacetic acid (TCA) in a final concentration of 10% and/or dissolution in 30% potassium hydroxide by a previously reported technique (4). Glucagon-I^{131} was generally present in too low a concentration to form a satisfactory precipitate by itself, but it was found that in the presence of 1 ml. of a 2% solution of dried normal human plasma or a 10% homogenate of rat liver, kidney, or muscle, 85–95% of the glucagon-I^{131} was precipitable with TCA. Therefore, TCA precipitable radioactivity was taken to be chiefly glucagon-I^{131}. Increases in the per cent of TCA soluble radioactivity were considered to represent degradation of the glucagon-I^{131} (5).

As the thyroid gland concentrates iodide far in excess of other tissues, it was necessary to perform special studies in order to evaluate the effect of this function upon the distribution of radioactive iodine following the administration of glucagon-I^{131}. Accordingly, one group of animals was prepared in the standard manner, while another group received 0.5% potassium iodide in the drinking water during the fasting period, and then 10 mg. of potassium perchlorate in 0.5 ml. of 0.9% saline, intraperitoneally, 1 hour prior to the experiment in order to suppress thyroidal accumulation of iodide (6).

Previous studies (4) have indicated that erythrocytes can acquire TCA soluble radioactivity by diffusion from the plasma after the administration of insulin-I^{131} to rats. Unless thorough washing is done prior to precipitation, this radioactivity will be occluded and appear as TCA precipitable material. Therefore, in the present studies, red cells were separated from heparinized whole blood, washed three times with two volumes of 0.9% saline, and hemolyzed prior to further processing.

Unless otherwise indicated, the tissue concentration of glucagon is expressed as the fraction of the dose per unit (gm. or ml.) of tissue×body weight (gm.). This corrects for differences in animal size and also allows comparison of the concentration in a given tissue to that in the total body (4).

Urinary and fecal excretion experiments were performed by injecting labeled hormone into the tail vein and then collecting the excreta at specified intervals for 48 hours. Urine was collected in iced beakers which contained 1 ml. of 300 mg.% bovine plasma albumin to serve as protein carrier in the precipitation of glucagon-I^{131}. Feces were obtained from the metabolic cage screens. Radioactivity in the specimens was determined after processing them in the same fashion as described above, and the accumulative excretion percentages calculated.

RESULTS

Distribution in rats. Each of eight rats was given 23 μg. of glucagon-I^{131} by tail vein and sacrificed 5 minutes later. Figure 1 shows the concentration of radioactivity in each of 25 tissues. In 7 tissues both the TCA precipitable and soluble radioactivity are shown. The 13 tissues which have a concentration of radioactivity greater than that in the total body are, in order of decreasing concentration: kidney, plasma, liver, pituitary, spleen, lung, salivary gland, adrenal, pancreas, thyroid, heart, duodenum, and lymph node. Twelve other tissues, including skeletal muscle, concentrate radioactivity to a lesser degree than would occur in the total body if the radioactivity were uniformly distributed. The brain, testes, and red blood cells localize negligible amounts of radioiodinated protein.

The upper part of the insert in Figure 1 shows that the concentration of radioactivity in the renal cortex greatly exceeds that in the medulla; it is nearly 5 times that in the plasma. Radioautographs suggest that this renal cortical localization is principally in the proximal convoluted tubules (7).

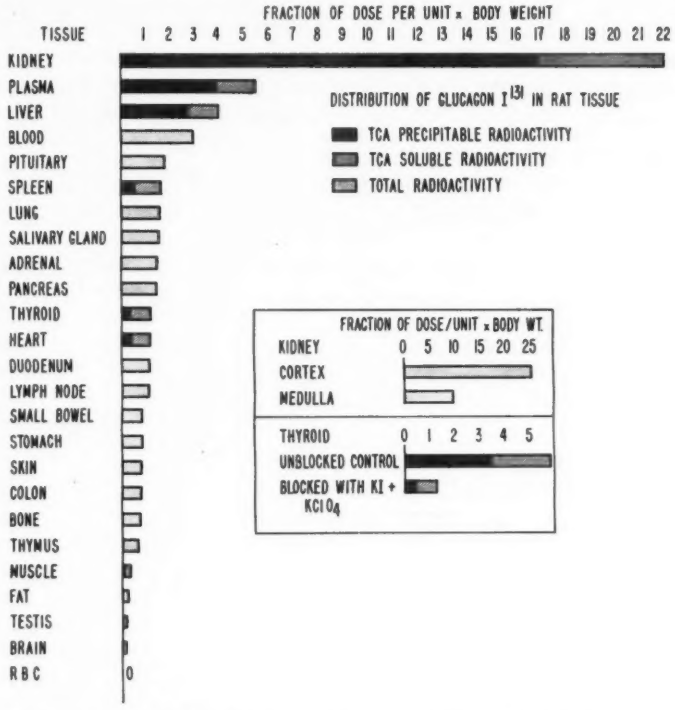

FIG. 1. All concentration values greater than unity represent localization in the specific tissue greater than the theoretical concentration in the total body.

The concentration of radioactivity in the thyroid is essentially the same as in plasma when the iodide "trapping" mechanism is not suppressed, as is indicated in the lower part of the insert of Figure 1. However, when the rats are treated with potassium iodide and potassium perchlorate, it is seen that most of the radioactivity which is concentrated by the unblocked gland is apparently iodide.

Considering the data in another fashion, the total (TCA precipitable and soluble) radioactivity in entire tissue masses, kidneys contain 14.9%, skeletal muscle[7] 11.9%, liver 11.7%, plasma[8] 8.2%, and the remainder of the tissues 51.3% of the radioactivity, 5 minutes after injection.

Urinary and fecal excretion. Each of 9 rats was given 33 μg. of glucagon-I^{131} by tail vein and placed in groups of 3 in metabolic cages. They were maintained without food, but with free access to water, for a period of 48

[7] Calculation based on the assumption that muscle mass constitutes 40% of the body weight.

[8] Calculation based on the assumption that plasma constitutes 2% of the body weight.

hours; excreta were collected at the times indicated in Figure 2. More than 40% of the total radioactivity leaves the body in the urine, but less than 0.6% of this is TCA precipitable. Less than 1% of the total dose is excreted in the feces in 48 hours. The figures clearly indicate that little undegraded glucagon leaves the body by these routes.

Changes in tissue distribution and degradation with time. Each of 24 rats was given 35 μg. of glucagon-I^{131} by tail vein, and groups of 6 animals each

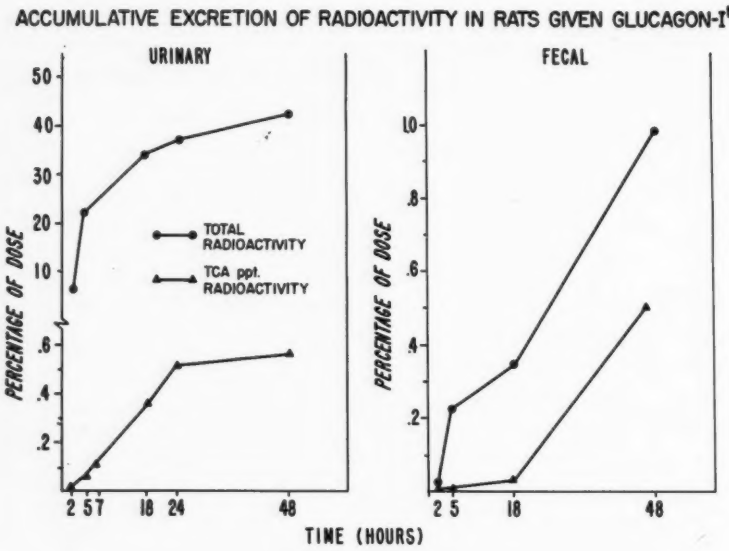

FIG. 2

were sacrificed after 5, 15, 30, or 60 minutes. Plasma, liver, kidney and muscle were assayed for TCA precipitable and soluble radioactivity. Figure 3A reveals that, especially in the first 15 minutes, kidney, plasma and liver concentrations of glucagon-I^{131} fall off rapidly whereas muscle levels change more slowly. In Figure 3B it is noted that muscle contains a particularly high percentage of TCA soluble radioactivity as early as 5 minutes after injection. This may indicate that TCA soluble radioactive material enters muscle more readily than does TCA precipitable material, but it also raises the possibility that muscle is extremely active in the degradation of glucagon-I^{131}. However, in contrast to the steady rise in the other tissues, the percentage of TCA soluble radioactivity in muscle shows little change in a period of 60 minutes following injection. This, and the low concentration of TCA precipitable radioactivity in muscle (Fig. 3A), suggest that muscle may participate less in the degradation of glucagon-I^{131} than do kidney and liver. The rise in plasma "degradation products"

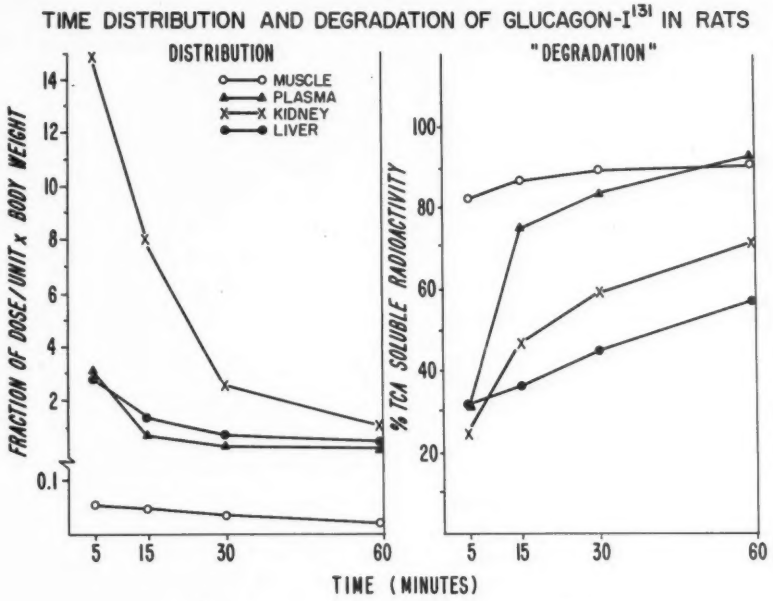

FIG. 3. Each point in Figure 3A represents the concentration of glucagon-I^{131} expressed as the precipitable fraction of the TCA precipitable dose per unit of tissue × body weight, present at the indicated time. This expression allows correction for the small amount of TCA soluble radioactivity present in the injected material. Figure 3B gives the percentage of TCA soluble radioactivity present in each tissue at the different times.

may represent diffusion of cleaved glucagon-I^{131} into the intravascular compartment.

Effect of removal of organs on plasma radioactivity after the injection of glucagon-I^{131}. Four groups of 12 rats each were operated on to provide laparotomy control, hepatectomized, nephrectomized and splenectomized preparations. Hepatectomy was performed by a previously reported technique (8), and nephrectomy and splenectomy were carried out by pedicle ligation and excision of the organs. Each animal was given 14 μg. of glucagon-I^{131} by tail vein and 24 animals were sacrificed at 5 minutes and the others at 15 minutes. Plasma was assayed for radioactivity and calculations were performed in the manner previously described.

Table 1 shows that a significantly slower fall in the concentration of TCA precipitable radioactivity in the plasma occurs when either the liver or kidney is excluded from the preparation. Splenectomy has no such effect. This provides additional evidence that kidney and liver are important in the degradation of glucagon-I^{131} in the rat.

Effect of portal venous injection on distribution. In each of 2 experiments

TABLE 1. THE EFFECT OF REMOVING CERTAIN ORGANS UPON PLASMA GLUCAGON-I^{131}

Treatment	Interval (mins.)	TCA precipitable radioactivity*	P value
Laparotomy control	5	4.7	
Hepatectomy	5	6.8	$<.001$
Nephrectomy	5	6.2	$<.01$
Splenectomy	5	4.8	$>.5$
Laparotomy control	15	1.3	
Hepatectomy	15	1.9	$<.01$
Nephrectomy	15	2.3	$<.001$
Splenectomy	15	1.4	$>.5$

* Fraction of dose/ml. $\times$ body weight in plasma.

8 rats were given labeled glucagon via the inferior vena cava and 8 via the portal vein. All were sacrificed 5 minutes after injection, and plasma, liver and kidney were assayed for radioactivity after treatment with TCA. In the initial experiment, each rat was given 4.5 μg. of glucagon-I^{131}, and in the second, 0.67 μg. of glucagon was injected. Figure 4 shows that at a given dosage level no differences in distribution or in the partition of radioactivity can be detected. The second study was performed to be sure that the higher dosage of the initial experiment did not obscure alterations

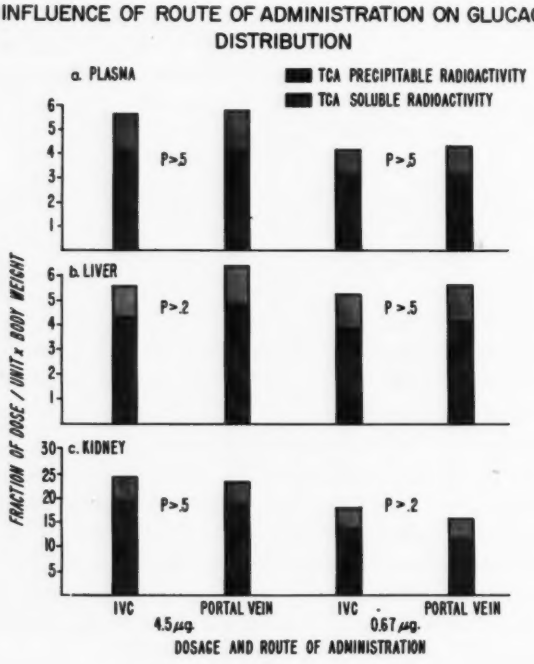

FIG. 4

of distribution and/or degradation that might be produced by portal venous administration.

DISCUSSION AND CONCLUSIONS

These data suggest that kidney and liver are important sites for the concentration and degradation of glucagon. The assumption that the radioactivity which is soluble in TCA is a measure of degraded glucagon is based principally on *in vitro* studies (5). Others have discussed this proposition in relation to insulin-I^{131} (4, 9, 10). It has been shown (5) that the increase in radioactivity in a TCA filtrate after incubation of glucagon-I^{131} with rat liver extract correlates with an increase in the optical density at 280 mμ. This suggests that proteolysis accompanies the changes in the radioactive label. Nevertheless, the percentage of TCA soluble material present in a given tissue may be affected by factors such as diffusion and adsorption, so that it cannot be taken as an exact index of the amount of degradation accomplished by that tissue *in vivo*.

Administration of glucagon-I^{131} via the portal vein does not alter its subsequent distribution or apparent degradation under the conditions of the study. This is in accord with the observation that kidney also plays a role in the inactivation of glucagon, but contrasts with the studies of Goldner (11) who, using a bioassay technique, concluded that a single passage through the liver effectively inactivated the glucagon in a commercial preparation of insulin.

A striking similarity exists between data from this study and those from work done with insulin-I^{131} (4), ACTH-I^{131} (12), prolactin-I^{131} (13), and ribonuclease-I^{131} (10). The distribution of human serum albumin-I^{131} and NaI^{131}, however, is quite different, which suggests that a common distribution is being observed for the radioiodinated proteins of smaller molecular weight. As a corollary, studies of this type may not be able to demonstrate increased or specific concentrations of hormones at their sites of physiological action. On the other hand, the high renal concentration which is consistently observed after the administration of labeled proteins of low molecular weight is noteworthy, and suggests that the kidney may play an important role in the metabolism of these substances. One possible explanation is that the proteins are filtered in the glomeruli and reabsorbed by the renal tubules, as suggested by Addis (14).

SUMMARY

Five minutes after injection of glucagon-I^{131} into the tail vein of rats, trichloroacetic acid precipitable radioactivity is found concentrated principally in kidney, liver and plasma. Radioactivity in the renal cortex is nearly 5 times greater than that in plasma. The concentration in skeletal muscle is small and changes relatively slowly. Hepatectomy or nephrectomy retards the fall in the concentration of trichloroacetic acid precipi-

table radioactivity in plasma at both 5 and 15 minutes. These observations suggest that kidney and liver are important sites of degradation of this hormone. Portal venous administration produces the same pattern of distribution and degradation as does injection by inferior vena cava, which suggests that the liver is not the only site of glucagon metabolism. The pattern of distribution and degradation of this hormone in the rat seem similar to that of other proteins of low molecular weight and may not be specifically related to sites of biological activity.

Acknowledgments

We are especially indebted to Miss Maureen O'Connell, Miss Ruth Miller, and Mrs. Barbee Tucker for valuable technical assistance.

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DEGRADATION OF GLUCAGON-I^{131} BY RAT TISSUES *IN VITRO*[1]

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IN A PREVIOUS communication (1) evidence has been presented that glucagon-I^{131} is rapidly degraded in rats and that this is a major metabolic fate of the material after intravenous administration. It was, therefore, of interest to investigate further the nature of this degradation.

MATERIALS AND METHODS

Crystalline glucagon was a gift of Eli Lilly and Company, and was kindly iodinated by Dr. Alfred Staub. The biological properties of this material have been described (1). Crystalline zinc insulin was also donated by Eli Lilly and Company.

Male Sprague-Dawley rats weighing 190 to 250 gm. were fasted for 18 hours and decapitated. Tissues were ground with a glass homogenizer in ice cold 0.067 *M* potassium phosphate buffer of pH 7.5. Heparinized aortic blood from stunned animals was used as a source of plasma. For cell fractionation studies, rat liver was homogenized in 0.25 *M* sucrose and separated into nuclear, mitochondrial, microsomal, and residual supernatant fractions by a modification (2) of the method of Strittmatter and Ball (3). Liver acetone powder was prepared for other experiments by homogenizing rat liver in 1.15% KCl and centrifuging for 20 minutes at $9000 \times g$. The supernatant material was then precipitated with 9 volumes of acetone at $-10°$ C, the precipitate was washed 5 times with acetone, and then dried *in vacuo* over concentrated H_2SO_4. Liver extract was prepared by stirring each 50 mg. of the powder with 1 ml. of ice cold distilled water for 1 hour, and then removing insoluble material by centrifugation.

In vitro assays of glucagon-I^{131} degradation were carried out by methods similar to those previously described for insulin-I^{131} (4). Two to 10 μg. of glucagon-I^{131} (usually about 0.5 μc.) were incubated in duplicate at 37° C in 2 ml. of 0.033 *M* potassium phosphate buffer with a homogenate or extract of the tissue to be studied. One mg. of crystalline bovine plasma albumin (Armour Laboratories) was added to each reaction mixture to help stabilize the glucagon; this did not affect the degradation of glucagon-I^{131} but did retard spontaneous alterations which may have been due to denaturation of the substrate. At appropriate intervals, the reaction was terminated by the addition of 3 ml. of 20% trichloroacetic acid (TCA). One ml. of 2% reconstituted dried human plasma was added just before the TCA to facilitate the precipitation of protein. The precipitate was washed twice with 10% TCA, and radioactivity in the supernatant and precipitate was measured in a well-type gamma counter. As a control, glucagon-I^{131} was incubated in buffer without the addition of tissue extract, and the TCA supernatant radioactivity,

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which amounted to about 3 to 12% of the total radioactivity, was subtracted from that of the experimental tubes.

In studies of proteolysis, 63 μg. of glucagon-I^{131} were incubated in 0.4 ml. of 0.033 *M* phosphate buffer at pH 7.5 containing 0.1 ml. of a liver acetone powder extract which had been dialyzed against phosphate buffer for 3 hours. The reaction was terminated at intervals by the addition of 0.6 ml. of 20% TCA and 0.2 ml of a 2% bovine plasma albumin solution to act as protein carrier. After filtration of the mixture the optical density of the filtrate was measured at 280 mμ against a blank containing phosphate, TCA, and water. Radioactive measurements of the TCA soluble and precipitating material were also made.

RESULTS AND DISCUSSION

Degradation of glucagon-I^{131} by various tissue homogenates. When 1 ml. of 0.4% homogenates of various rat tissues were incubated for 5 minutes with 2.4 μg. of glucagon-I^{131}, the results shown in Table 1 were obtained. It is seen that all of the tissues examined, except plasma, exhibited an appreciable ability to degrade glucagon-I^{131}. When the distribution of glucagon-I^{131} *in vivo* (1) and the total organ masses are considered, however, liver, kidney, and muscle appear to be especially important sites of degradation.

TABLE 1. DEGRADATION OF GLUCAGON-I^{131} BY RAT TISSUE HOMOGENATES

Tissue	% of radioactivity in TCA supernatant in 5 minutes
Liver	49
Kidney	42
Testis	37
Thymus	30
Spleen	28
Lymph node	26
Gastrocnemius muscle	11
Plasma	<1

Relation of radioactive changes to proteolysis of glucagon. Figure 1 shows that when glucagon-I^{131} was incubated with rat liver acetone powder extract, there was an increase in TCA soluble radioactivity, and that this was paralleled by an increase in the optical density of the TCA filtrate at 280 mμ. Since the change in optical density can be taken as a measure of proteolysis, this suggests that the increase in TCA solubility of the radioactive label is associated with degradation of the glucagon molecule.

Further support for this concept was obtained in the following experiment. 2.1 μg (5×10^{-4} μmoles) of glucagon-I^{131} were incubated with 0.004 ml. of liver acetone powder extract. When non-labeled glucagon was added to the reaction mixture, it was found, as shown in Table 2, that the rate of degradation of the iodinated material was depressed. This suggests that the nonlabeled material was competing with the labeled compound as substrate for a proteolytic enzyme system. An effect of nonlabeled glucagon upon a deiodinating mechanism would appear less likely. It is seen that the addition of labeled glucagon was slightly more effective than the addition

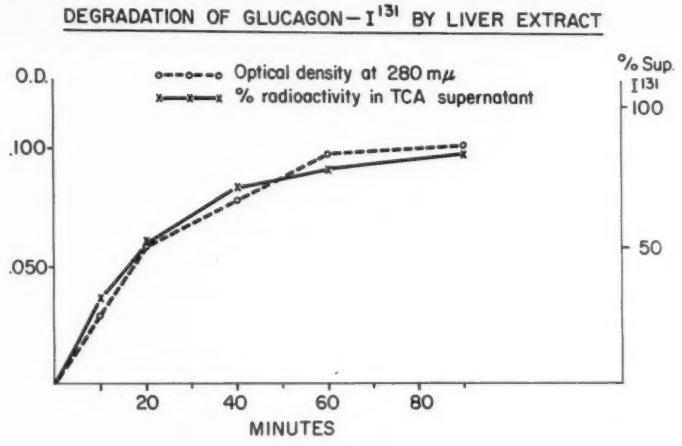

FIG. 1

of equivalent amounts of the nonlabeled material in suppressing the percentage degradation of the iodinated material. It is possible that the manipulations involved in iodination caused slight changes in the glucagon molecule which rendered it a better substrate for proteolysis. The marked depression of the degradation of glucagon-I^{131} which occurred when insulin was added is of of interest in view of the finding of Tomizawa and Williams (5) that nonlabeled glucagon competes with insulin-I^{131} for degradation by a liver enzyme system. With the crude liver preparations employed, it is not possible to rule out the possibility that degradation products, rather than the proteins themselves, are acting as competitive substrates. The observation that insulin can protect glucagon-I^{131} from rapid degradation by a liver extract may help to explain the augmentation of the glycogenolytic action of glucagon by insulin that was noted by Tyberghein *et al.* (6) *in vitro*.

TABLE 2. EFFECT OF VARYING AMOUNTS OF GLUCAGON AND INSULIN UPON RATE OF DEGRADATION OF GLUCAGON-I^{131} BY LIVER EXTRACT *in vitro*

μmoles of substrate added			% of radioactivity in TCA supernatant in 5 minutes
Glucagon-I^{131}	Glucagon	Insulin	
2.5×10^{-4}			16.5
5×10^{-4}			12.2
10×10^{-4}			7.2
20×10^{-4}			3.9
5×10^{-4}	5×10^{-4}		10.2
5×10^{-4}	10×10^{-4}		8.6
5×10^{-4}	15×10^{-4}		6.7
5×10^{-4}		5×10^{-4}	2.3
5×10^{-4}		10×10^{-4}	1.6
5×10^{-4}		20×10^{-4}	0.5

Molecular weights were taken as 4000 for glucagon and 6000 for insulin

The fact that rat liver extract does not appear to degrade radio-iodinated human serum albumin at pH 7.5 has previously been suggested as additional evidence that direct deiodination of intact protein is not an explanation for the changes in TCA solubility of the radioactive label (4).

The maximum degradation of glucagon-I^{131} that was noted in any experiment corresponded to about 90% radioactivity in the TCA supernatant. Under conditions where maximal changes in the TCA solubility of the radioactive label occurred, it was found that the glucagon-I^{131} was biologically inactivated.[3]

Thus, there is considerable evidence which suggests that the changes in TCA solubility of glucagon-I^{131} produced by liver extract *in vitro* are associated with proteolysis rather than direct deiodination of the intact molecule.

Enzyme characteristics and cytoplasmic distribution of the glucagon-I^{131} degrading system of liver. The effect of variation of pH upon the ability of rat liver extract to degrade glucagon-I^{131} was studied in potassium phosphate buffer in the pH range of 5 to 8. Maximum activity was observed at around pH 7 to 7.5; at lower pHs activity fell off markedly, and at pH 5 it was less than 20% of the maximum. The degradative system did not lose appreciable activity when dialyzed against phosphate buffer for 24 hours, but was completely inactivated when exposed to a boiling water bath for 10 minutes. The sulfhydryl blocking agent, *p*-chloromercuribenzoic acid, in a concentration of 5×10^{-5} *M* also completely inhibited the ability of 0.02 ml. of liver extract to degrade glucagon-I^{131} in a 2 ml. reaction mixture. This suggests that sulfhydryl groups are necessary for the activity of the enzyme system.

Rat liver cytoplasmic fractions obtained by differential centrifugation in 0.25 *M* sucrose were adjusted to pH 7.5 and diluted to a 0.3% tissue concentration. One ml. of each fraction was incubated with 10 μg. of glucagon-I^{131} in phosphate buffer for 5 minutes. Under these conditions the rate of degradation was directly proportional to the concentration of homogenate. In Table 3 are presented averages of the results from two experiments which were performed in duplicate on livers from a total of 6 rats. Most of the activity of the whole homogenate was found in the residual supernatant fraction. Furthermore, most of the activity remained soluble when the ribonucleic acid of the residual supernatant fraction was precipitated with acetic acid at a pH of 5 as recommended by Griffin *et al.* (7). This suggests that the hepatic enzyme system which degrades glucagon-I^{131} is not firmly or principally bound to structures rich in nucleoprotein. Gianetto and de Duve (8) have demonstrated that certain enzymes, including a cathepsin of rat liver with maximum activity at an acid pH, exhibit appreciable activity only after procedures such as re-

[3] Kindly performed by Dr. Jean M. Tyberghein, using a liver slice method of *in vitro* bioassay.

TABLE 3. DISTRIBUTION OF GLUCAGON-I^{131} DEGRADING ACTIVITY IN CYTOPLASMIC FRACTIONS OF RAT LIVER

Fraction	% of homogenate activity*
Nuclear	6
Mitochondrial	6
Microsomal	4
Residual supernatant (S-3)	86
pH 5 precipitate of S-3	12
pH 5 supernatant of S-3	60
Mitochondria after freeze-thawing	8
Microsomes after freeze-thawing	3

* Activity of each fraction was determined in terms of % radioactivity in TCA supernatant after incubation for 5 minutes. This was then expressed as the % of the activity of whole liver homogenate which was simultaneously assayed.

peated freezing and thawing which disrupt the particles with which the enzymes are associated. In the present studies it was found that rapidly freezing and thawing the mitochondrial and microsomal fractions 10 times in succession did not greatly alter their activity in degrading glucagon-I^{131}. This is further evidence that the degradative system which acts on glucagon-I^{131} may exist in the cytoplasm in a soluble form.

SUMMARY

Glucagon-I^{131} can be degraded by homogenates of various rat tissues. Liver and kidney are among the more active tissues in this respect. Evidence is presented which suggests that the increase in TCA solubility of the radioactive label that occurs when glucagon-I^{131} is incubated with liver extracts is due to proteolysis rather than to direct deiodination. Insulin and glucagon appear to compete as substrates for a degradative enzyme system of liver. The liver enzyme system which degrades glucagon-I^{131} is present principally in the residual supernatant fraction of cytoplasm.

Acknowledgment

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THE EFFECT OF TESTOSTERONE PROPIONATE UPON INCORPORATION OF LABELED GLYCINE INTO MOUSE KIDNEY SLICES[1,2]

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ONE of the characteristic properties of androgenic steroids generally is their ability to promote protein synthesis in certain tissues other than those of the male reproductive tract. The gross manifestations of this anabolic property have been described in detail (cf. 1). However, detailed knowledge of the mechanisms involved is almost totally lacking. Selection of *in vitro* incorporation studies as a tool for the investigation of this problem is based upon repeated observations that a direct correspondence exists between the rate at which labeled amino acids are incorporated into protein *in vitro* and increased protein anabolic activity in various physiological situations. Thus, increased incorporation rates are well known to characterize rapidly growing foetal or regenerating adult liver. Similar increases have been reported to accompany the growth-promoting action of estrogens upon rat uteri (2, 3) and the acquisition of secretory competence by endocrine tissues (4). We have previously shown that the action of estrogen and relaxin, separately or in combination, in stimulating growth of the guinea pig symphyseal ligament is accompanied by a 2- to 5-fold increase in rate of incorporation of labeled glycine into symphyseal connective tissue *in vitro* (5) as well as *in vivo* (6).

The experiments to be described deal with the effects of testosterone propionate upon the incorporation of carboxyl-labeled glycine into mouse kidney slices.

EXPERIMENTAL

A/Jax mice were obtained from the Jackson Laboratories or bred at Tufts from Jackson Laboratory stock. Testosterone propionate[3] (T.P.) was injected subcutaneously in 0.1 ml. sesame oil. Control mice were either uninjected, or received the same volume of sesame oil. Purina rat chow was fed *ad lib.* except as noted below. The animals were

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[2] Some of this material was presented at the April, 1956 meeting of the American Society of Biological Chemists, Atlantic City, New Jersey.

[3] Neohombreol (Organon).

killed by a blow on the head, tissues were removed, weighed rapidly on a Roller-Smith balance, and sliced (except for diaphragm) with a Stadie-Riggs microtome. Slices from each kidney (80–150 mg.) were usually suspended separately in 2.0 ml. of Krebs-Ringer phosphate containing glycine-1-C^{14}. The flasks were incubated, with shaking, at 38.5° C with air as the gas phase. Unless otherwise indicated, the glycine concentration was 1.0 mM, and its specific activity was about 0.03 micro-curies/micro-mole (approximately 40,000 cpm/ml.).

At the end of the incubation period (usually 2 hours) the flasks were withdrawn from the bath and placed on ice. The tissue slices were removed, blotted on filter paper, and then homogenized in a total of three ml. water. The proteins were precipitated by the addition of TCA to 5%; the precipitate was redissolved in dilute NaOH and the precipitation was repeated twice, the last time in the presence of inert glycine (1%). The precipitated protein was washed successively with acetone, chloroform:ether:ethanol (1/1/2) and benzene, then plated from benzene suspension. The dried protein was counted in a windowless flow counter, the data being corrected to uniform sample thickness (5 mg./cm.)[4] from a self-absorption curve obtained with radioactive plasma protein. From the corrected specific activity, and the concentration of total and labeled glycine in the medium, the average incorporation rate (expressed as micrograms of glycine carbon taken up per gram of protein per hour) was calculated.

RESULTS

The choice of A/Jax mice for these experiments was based upon the work of Fishman, *et al.* (7) who demonstrated that the action of testosterone propionate in this strain was characterized by a large increase in kidney β-glucuronidase. Conditions chosen for initial exploratory experiments were also based upon their work. In Table 1 are summarized the results of two experiments in which incorporation rates for kidney and other tissues were measured after three injections, at 48-hour intervals of 1.0 mg. T.P.

Table 1 indicates that the anabolic action of T.P. is reflected in a clear increase in rate of incorporation into kidney slices, an effect not shared by the other tissues studied. Under these conditions, quantitatively identical effects were exerted by T.P. upon kidneys of both males and females.[3] Females were used for the rest of the experiments to be reported.

The calculation of a figure for the average uptake rate during an incorporation experiment involves the tacit assumption that the incorporation process is linear during the period of the experiment, and that no lag period exists. Figure 1 shows this to be the case; for both control and treated mice incorporation is linear during at least three hours' incubation.[5]

[4] For both sets of experiments included in Table I, the differences between control and T.P.-treated groups was highly significant ($p < 0.01$). In general, the standard errors of the means for a group of mice, whether treated or not, ranged from three to a maximum of ten per cent of the mean. Differences between group means were considered significant, therefore, if they exceeded 15–20% of the control value ($p < 0.05$).

[5] It will be noted that the interpolated value for two hours' incubation from Figure 1 is 0.55 micro-moles incorporated/gm. of protien. The corresponding rate is therefore

TABLE 1

	Males		Females	
	Controls (6)*	T.P.-Treated (7)	Controls (7)	T.P.-Treated (8)
Kidney Weight (% of body wt.)	1.53	2.05	1.25	1.56
Incorporation Rate (μmoles/gm./hr.)				
Kidney	0.38 ± .04**	0.53 ± .03	0.40 ± .02	0.58 ± .03
Diaphragm	0.13 ± .014	0.15 ± .016	0.11 ± .009	0.11 ± .011
Liver	0.20 ± .026	0.17 ± .020		
Skeletal Muscle	0.014 ± .0035	0.014 ± .0017		

* Numbers in parentheses indicate number of mice used. Each kidney was sliced and incubated separately.

** Average ± Standard error.

In Figure 2, the per cent increase in incorporation rate is plotted as a function of the duration of the experiment (i.e., the time elapsing between the first injection of T.P. and sacrifice). Except for the data at 13 and 36 hours, T.P. injections were repeated at 24-hour intervals, and the animals were killed 24 hours after the last injection. The experiment at 13 hours was terminated 13 hours after a single injection; that at 36 hours

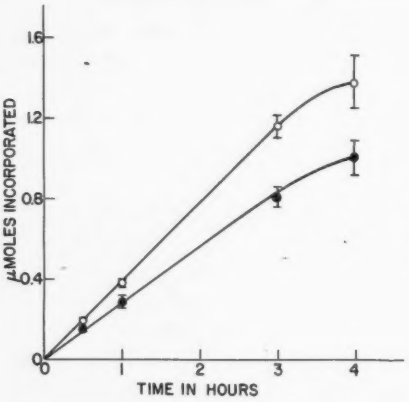

FIG. 1. Effect of time of incubation upon incorporation into kidney slices from control (—●—) and T.P.-treated (—○—) mice. Horizontal lines are drawn one S. E. above and below each point.

0.275 micro-moles/gm./hr., a figure which is substantially (30%) less than the corresponding figure in Table I. Large variations in measured incorporation rates for untreated mice have frequently been observed during the eighteen months covered by these observations. These variations could not be referred to the influence of age, diet or endocrine status, nor could consistent differences be observed between mice obtained from Bar Harbor and those bred locally. Values obtained during the summer months were usually lower, and those in the fall somewhat higher than the average, but no definite conclusions could be drawn about this point. Consequently, for each experiment to be reported, the data are compared to a similar control group run at approximately the same time.

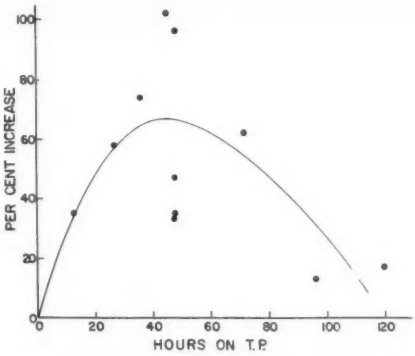

FIG. 2. Variation of incorporation rate with length of treatment with T.P. (For details, see text.)

represents two injections 18 hours apart, the experiment being concluded 18 hours after the second.

It may be seen that the effect of testosterone propionate reaches a maximum after two injections 24 hours apart and declines upon further, repeated treatment. Accordingly, in the other experiments to be described, 48 hours was chosen as the optimal time for the duration of the experiment.

The amount of T.P. required to exert a maximal effect appears to be far less than 1 mg./day. Figure 3 shows that 0.1 mg. administered daily, produced as great an increase as did 5.0 mg. The protein anabolic effect, as measured by kidney weight, is also as pronounced at the lower as at the higher dose.

Two experiments were performed to assess the effect of nutritional status upon the response, since studies by Fishman, *et al.* (8) and by Homburger and Pettengill (9) have emphasized the importance of this factor upon response to androgenic stimulation of kidney β-glucuronidase and kidney weight, respectively. In the first experiment, 2.0 mg. of T.P. was administered to a group of six mice in two injections during 48 hours. Food was withdrawn from this group, as well as from a group of uninjected controls for the last 24 hours of the experiment, and incorpora-

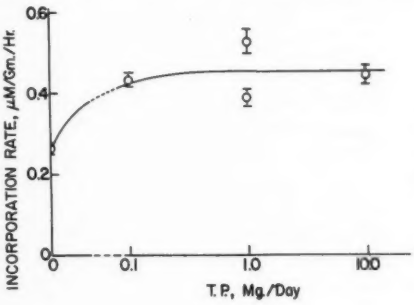

FIG. 3. Response of mouse kidney slices to treatment with various amounts of T.P. Incorporation rate is in micromoles of glycine carbon incorporated per gram of protein per hour.

TABLE 2. EFFECT OF TESTOSTERONE PROPIONATE UPON INCORPORTION OF GLYCINE-1-C^{14} INTO KIDNEY SLICES FROM UNFED MICE

	Starved 24 hr.		Fed*
	No. of Mice**	Incorporation Rate† (μM/gm. protein/hr.)	Incorporation Rate
Controls	6	0.32 ± 0.01	0.43
T.P.-Treated	6	0.49 ± 0.03	0.55

* These data were interpolated from another experiment relating incorporation to glycine concentration.
** Each kidney was sliced and incubated separately.
† Average ± S.E.

tion rates measured as before. The results are shown in Table 2. (For comparison, corresponding values for fed mice, interpolated from a curve relating glycine concentration and incorporation rate which was determined at the same time are included). Incorporation into kidney slices is diminished slightly by starvation, but the increase in response to T.P. (53%) is not appreciably affected.

In the second experiment, a number of mice were placed on a 25% protein, 25% fat diet (8). At two-day intervals, incorporation into kidney, liver, and diaphragm was measured. On the ninth and tenth days, half the remaining mice received 1.0 mg. T.P. Figure 4 shows that the effect of this regime was a transitory increase in incorporation rate in all tissues studied, with the greatest increase being manifested in liver. Under these conditions, the response of kidney slices to T.P. (44%) was within the range of values previously found. As was to be expected, incorporation into liver and diaphragm was unaffected by T.P.

The intracellular distribution of incorporated activity, and the effect of T.P. treatment upon the distribution, was also studied. For this experiment, both kidneys of each mouse were sliced and incubated in the same flask. After two hours' incubation, the slices were removed, homogenized in 0.25 *M* sucrose, and fractionated into nuclear, mitochondrial, and supernatant (including microsomes) fractions according to the method of

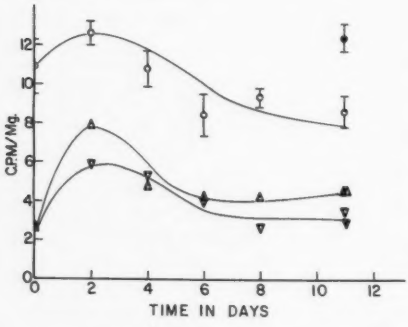

FIG. 4. Effect of 25% protein-25% fat diet upon incorporation into slices of kidney (—○—), liver (—△—) and diaphragm (—▽—) of A/Jax mice. Ordinate: CPM/mg. protein; Abscissa: time on diet in days. Filled symbols indicate data obtained with T.P.-treated animals. For the kidney slice data, horizontal lines bracket one standard error above and below each point.

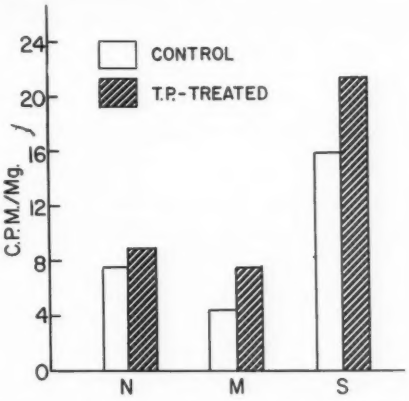

FIG. 5. Distribution of incorporated activity among various particulate fractions in treated and untreated A/Jax mice. N: Nuclei; M: Mitochondria; S: Supernatant (including microsomes). Ordinate: Counts per minute per mg. precipitated protein.

Schneider and Hogeboom (10). The protein in each fraction was precipitated and counted as before. The distribution of radioactivity among the three fractions for groups of control and T.P.-treated mice is compared in Figure 5. Although the specific activity of all fractions was elevated in the treated mice, the greatest increase (70%) was in the mitochondrial fraction.

In order to gain further insight into the mechanism of the T.P. effect, the effect of changing the glycine concentration in the incubation medium was studied. Three experiments were performed. In each, slices from control and treated (1.0 mg./day for two days) animals were incubated in a series of media in which the total glycine concentration was varied (by the addition of inert glycine) over a wide range. The data (Fig. 6) have been expressed as per cent increase in the treated group compared to tissues from untreated animals, and plotted against the logarithm of the glycine concentration. Included in Figure 6 is the average increase found at 1.0 mM glycine found in several other experiments (filled circle). Despite considerable scattering, it would appear that the maximum effect of T.P.

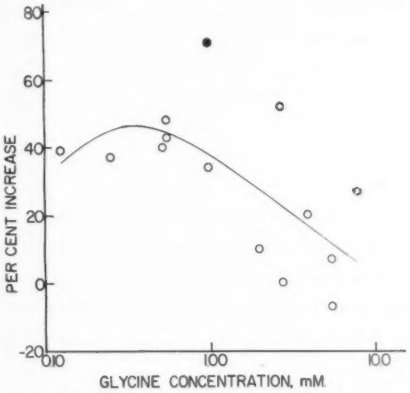

FIG. 6. Effect of glycine concentration of medium upon response to T.P. Per cent increase over controls vs. concentration of glycine in millimoles/liter.

is displayed at glycine concentrations 1.0 mM and lower, and that the effect, in general, diminishes at higher levels of substrate.

DISCUSSION

It is of interest to compare the effect of T.P. upon incorporation rate with its concomitant renotropic effect, i.e., increase in kidney weight. With A/Jax females, the total kidney weight in untreated animals averages about 1.25 gm. per 100 gm. body weight. Upon treatment with T.P., this slowly increases to 1.40–1.50 gm./100 gm. body weight, reaching a plateau from the 60th to the 120th hour. Significant increases in incorporation rate occur considerably sooner than do obvious weight changes. The well-known "wearing-off" effect of anabolic agents has its counterpart in incorporation phenomena, since after 120 hours of daily treatment, the incorporation rate is not significantly higher than in controls.

There appears to be no correlation between kidney size, relative to total body weight, and measured incorporation rate, nor does there appear to exist a simple relationship between response of individual kidneys to T.P., as measured by weight increase, and incorporation rate.

The experiments dealing with incorporation at various glycine concentrations were based upon the belief that the locus of action of biologically active steroids, in view of their solubility properties, might logically be expected to be at the cytostructural interface; it is reasonable to expect, therefore, that experiments designed to exhibit effects upon intracellular transport mechanisms might be rewarding. The data of Figure 6 suggest that T.P. acts, in part at least, by facilitating the intracellular accumulation of glycine from a relatively glycine deficient medium. Saturation of the concentrative mechanism by excess glycine would be expected to reduce or abolish the effect, as was, in fact, observed.

These observations suggest that *in vitro* incorporation phenomena may provide a valuable tool for the study of the mechanism of the protein anabolic action of steriod and other hormones. In particular, comparison of the response, as measured by incorporation studies, of reproductive tract tissue with parenchymal tissues might shed light not only upon the mode of action of androgenic substances per se, but also upon specificity of response to steriods in general.

SUMMARY

It is possible to demonstrate an increase in the rate of uptake of labeled glycine in kidney slices of A/Jax mice treated with testosterone propionate. The effect is independent of incubation time for at least three hours' incubation, and appears to reach a maximum some 48 hours after initiating daily treatment with the steriod. One-tenth milligram of T.P. is as effective as 50 times this dose.

Experiments in which the concentration of glycine in the medium was varied suggest that one of the mechanisms involved may be a facilitation of the intracellular accumulation of glycine from a relatively glycine-deficient medium, since the effect of T.P. tended to disappear at glycine concentrations greater than 1.0 mM.

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PANTOTHENIC ACID AND ADRENOCORTICAL HORMONE SECRETION[1]

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ATTENTION was first drawn to the relationship between panthothenic acid and the adrenal cortex by the experiments of Daft and Morgan (1, 2), in which it was demonstrated that adrenal hypertrophy, hemorrhage, and necrosis were prominent manifestations of pantothenic acid deficiency in animals. These observations were confirmed and extended by Deane and McKibbin (3) who found, using histochemical techniques, that the adrenal cortex of pantothenate-deficient animals has a reduced content of lipids, sudanophilic material, and ketosteroid. In addition to structural alterations, recent studies by Winters (4, 5, 6), Hurley and Mackenzie (7), and Dumm (8) suggest that there is also functional impairment of the adrenal cortex in pantothenate-deficient rats. Although these investigations have indicated that there is diminished secretion of steroid hormones by the adrenals of deficient animals, cortical hormone production was measured indirectly. The purpose of the study reported herein was to assess the secretory ability of the adrenal cortex of pantothenic acid deficient rats by direct measurement of steroid hormone secretion. The results of these experiments demonstrate that, in pantothenic acid deficiency, the secretion of steroid hormones by the isolated rat adrenal cortex is decreased.

METHODS

Weanling, male, Sprague-Dawley rats weighing 35–40 gm. were placed in three experimental groups. Animals in Group I were fed a synthetic diet deficient in pantothenic acid (9). Rats in Groups II and III consumed a similar diet which was fortified with one milligram of pantothenic acid per 100 gm. of food. Group II animals were pair fed with the deficient group while those in Group III were fed *ad libitum*. All groups were maintained on the diets for approximately 35 days. At the end of this period, animals were sacrificed and the adrenals quickly removed, dissected free of surrounding fat, bisected, and weighed. Each adrenal was placed in a beaker containing 2 ml. of Krebs-Ringer-phosphate solution, pH 7.4, to which one unit of ACTH was added. The Krebs-Ringer-phosphate solution contained 200 mg. % glucose. The adrenals were incubated aerobically for two hours at 37° C in a Dubnoff Metabolic Incubator. On completion of incubation, the steroids secreted by the isolated rat adrenals were recovered and quantitatively determined by a method which has been previously described (10).

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RESULTS

Animals fed the deficient diet consumed less food throughout the experimental period than did the *ad libitum* fed controls. This was reflected by a diminished growth rate which was apparent by the end of the first week. The vitamin deficiency also adversely affected the rate of growth since the pair fed control animals weighed considerably more at the end of the experimental period than did the deficient group (Table 1). Other signs of pantothenic acid deficiency became evident after the animals had been on the diet for 20–25 days. These consisted of prophyrin staining of the hair, loss of fur from the head and neck, body tremors, and sudden death.

TABLE 1. BODY WEIGHTS AND ADRENAL WEIGHTS OF VARIOUS GROUPS AFTER FIVE WEEKS ON EXPERIMENTAL DIETS
(Mean Values ±S.E. of the Mean)

	Body weight (grams)	Adrenal weight (mg.)	Relative adrenal weight (mg./100 grams body weight)
Group I Pantothenic Acid Deficient Animals (25)*	62.3 ±5.12	23.4 ±1.5	38.0 ±1.9
Group II Pair Fed Animals (18)*	108.0 ±3.0	24.4 ±1.0	22.8 ±1.0
Group III *Ad Lib.* Fed Animals (10)*	184 ±4.3	28.8 ±1.3	15.7 ±2.4

* Indicates the number of animals in each group.

Adrenal weights of the deficient, pair fed, and *ad lib.* fed animals are presented in Table 1. The actual mean adrenal weight of the deficient group was almost the same as that of the pair fed controls and less than that of the *ad lib.* fed animals. The relative adrenal weight of the deficient rats (expressed as mg. of adrenal tissue per 100 gm. body weight) demonstrates, however, that marked adrenal hypertrophy occurred in these animals. Gross hemorrhages were present in the adrenals of several of the deficient group.

The secretion of steroid hormones by the isolated adrenals was measured by a spectrophotometric procedure and by the Porter-Silber method (11). The spectrophotometric procedure detects steroid compounds which possess an α, β, unsaturated ketone structure in ring A of the molecule. The Porter-Silber method measures only those steroids which contain a 17,21-dihydroxy-20-ketone side chain. It has been previously demonstrated in normal animals that the steroids secreted by the isolated adrenal and which are detected by the spectrophotometric assay amount to more

than twice the quantity of hormones which are measured by the Porter-Silber reaction (10).

Actual cortical hormone secretion, as measured by both methods, was significantly reduced in the deficient animals as compared to the pair fed and *ad libitum* fed control rats (Table 2). Because of the variation in mean adrenal weights among the three groups, the steroid secretion is also presented as micrograms of steroid secreted per 100 mg. of adrenal tissue. When the values are expressed in this manner, the production of hormones by the adrenals of the deficient animals is significantly less than that of the pair fed group. Adrenal hormone secretion of the deficient group is also less than that of the *ad lib.* fed control animals when measured by the spectrophotometric method, but when determined by the Porter-Silber procedure there is no significant difference.

TABLE 2. STEROID SECRETION BY THE ISOLATED RAT ADRENAL GLAND
(Mean Values ± S.E. of the Mean)

	Spectrophotometric determination (Micrograms of steroid secreted per adrenal)	Porter-Silber determination (Micrograms of steroid secreted per adrenal)	Spectrophotometric determination (Micrograms of steroid secreted/100 mg. adrenal tissue)	Porter-Silber determination (Micrograms of steroid secreted/100 mg. adrenal tissue)
Group I Pantotheñic acid Deficient Animals (25)*	4.68 ± .38	2.57 ± .18	40.1 ± 3.05	22.8 ± 1.79
Group II Pair Fed Animals (18)*	7.67 ± .57[a]	3.57 ± .32[a]	62.7 ± 4.0[a]	29.2 ± 1.97[b]
Group III *Ad Lib.* Fed Animals (10)*	9.66 ± .93[a]	3.50 ± .34[b]	67.3 ± 3.9[a]	24.6 ± 2.3[c]

[a] Difference from Group I statistically significant, P = <0.01
[b] Difference from Group I statistically significant, P = <0.05.
[c] Difference from Group I not statistically significant
* Indicates the number of animals in each group.

DISCUSSION

It has been suggested by previous investigators that adrenal cortical hormone secretion is diminished in pantothenic acid deficient animals. In these studies, cortical hormone production was measured by indirect methods such as depletion of circulating eosinophils following administration of ACTH and epinephrine (4), increased sensitivity to insulin (5), alterations in adrenal cholesterol concentration (6), and changes in carbohydrate metabolism in response to stress (7). Other investigators (12, 13) using similar techniques, have disagreed with the conclusion that dimin-

ished adrenocortical function occurs in animals deficient in this water-soluble vitamin.

In the studies described herein, an attempt has been made to more clearly define the relationship between pantothenic acid and adrenocortical function. The ability of the isolated adrenal cortex of pantothenic acid deficient rats to secrete steroid hormones under the stimulus of ACTH has been measured directly. The results demonstrate that adrenocortical hormone secretion is diminished in pantothenic acid deficiency and thus confirm previous observations which indicated that pantothenic acid is essential to maintain the functional integrity of this gland.

Since it has been shown that pantothenic acid is essential for elaboration of adrenocortical hormones in response to ACTH, the possible mechanism by which this vitamin facilitates steroid hormone secretion must be sought. It is known that pantothenic acid does not exist in the free state in the animal body and that the metabolically active form of the vitamin is coenzyme A. Coenzyme A functions as an intracellular carrier of acetyl groups and is necessary for the condensation of these two carbon fragments in the formation of fats, certain amino acids, carbohydrates, cholesterol, and steroids. The primary reaction leading to the formation of these complex substances from acetate is the condensation of two molecules of acetyl-coenzyme A to form aceto-acetyl coenzyme A. The exact chain of reactions leading to the formation of the steroid molecule from this compound is not known, but it is presumed that further condensations take place. Thus, since pantothenic acid is an integral part of coenzyme A, a deficiency leads to depletion of body stores of this coenzyme. It seems reasonable to assume that the end result will be a failure of synthesis of complex substances including the adrenal steroid hormones.

SUMMARY

Steroid secretion by the isolated adrenal glands of pantothenate-deficient, pair fed, and *ad libitum* fed rats has been determined.

The results demonstrate that the secretion of adrenocortical hormones is diminished in pantothenic acid deficient animals as compared to pair fed and *ad libitum* fed controls.

It is suggested that diminished adrenal steroid synthesis in pantothenate-deficient rats results from depletion of body stores of coenzyme A.

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EFFECT OF FASTING ON INSULINASE ACTIVITY AND ON HYPOGLYCEMIC RESPONSE TO INSULIN[1]

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THE inactivation and degradation of insulin by homogenates of rat livers are decreased when such homogenates are prepared from livers of fasted rats (1, 2). Slices of livers from fasted rats, however, show an increase in the rate of inactivation of insulin (3). Since neither homogenates nor slices of liver necessarily reflect the actual state of physiological processes, it became pertinent to determine the influence of fasting on the rate of insulin destruction by intact animals and thereby establish which of the above findings are of physiological significance. Further, since a change in the rate of destruction of insulin may result in a change in the hypoglycemic response to a standard dose of exogenous insulin, the effect of fasting on the response of rats and mice to insulin was determined.

METHOD

Male mice, weighing from 20 to 30 gm., were fasted for various periods. At the end of a designated period, the mice were given an intraperitoneal injection of 0.4 units insulin in 0.02 ml. acidified water per gram of body weight and a subcutaneous injection of 1 ml. of a 5.5% glucose solution. The insulin consisted of a mixture of crystalline zinc insulin and tracer quantities of I^{131} labeled insulin. All mice were sacrificed one hour after the injection of the insulin. The preparation of the animals and the determination of the quantity of insulin destroyed per hour per 100 gm. of mouse was the same as that described in a previous report (4).

The hypoglycemic response to exogenous insulin was determined in groups of male rats of the Carworth strain weighing from 150 to 300 gm. and groups of male mice of from 20 to 30 gm. in weight. After a designated period of fasting the rats were given an intraperitoneal injection of 0.05 unit insulin in 0.2 ml. acidified water per 100 gm. of body weight while the mice were given an intraperitoneal injection of 0.0025 unit insulin in 0.02 ml. acidified water per gram of body weight. Blood samples were taken from the clipped tail into 0.1 ml. pipettes immediately before and at stated intervals after the injection of insulin. The glucose concentration of the blood was determined by Nelson's procedure (5). Throughout these tests the rats and mice were relatively immobilized in wire containers.

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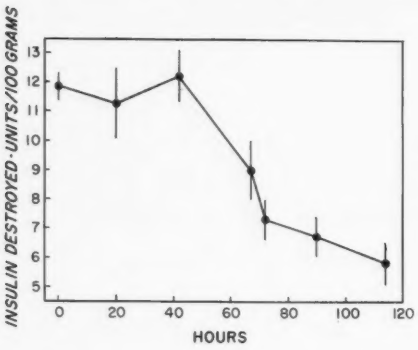

FIG. 1. The Effect of Fasting on the Destruction of Insulin by Intact Mice. The insulin destroyed at each period of fasting is plotted as units (mean ±SE) per hour per 100 grams.

RESULTS

The effect of various periods of fasting on the destruction of insulin by the intact mouse is illustrated in Figure 1 which depicts the mean (±SE) units of insulin destroyed per hour per 100 gm. at the designated period by groups of from 10 to 20 mice. It is apparent that no significant change in the rate of insulin destruction occurs until the mice have been fasted for more than 42 hours; thereafter, the decrease in the quantity of insulin destroyed per hour is statistically highly significant ($p < 0.001$). It is pertinent to note that many mice do not survive a fast of more than 96 hours and consequently larger groups than were actually tested were fasted in order to have from 10 to 20 animals for each period of fasting.

The effect of fasting on the hypoglycemic response of mice and rats to exogenous insulin is summarized in Tables 1 and 2 respectively. In order to permit comparison between the responses of groups of animals subjected to different periods of fasting and between the responses of the two species, the mean blood sugar concentration for each interval after the administration of insulin was expressed as the per cent of the initial concentration (Fig. 2). The data reveal that there is a significant decrease ($p < 0.001$) in the blood sugar concentration by the end of the first day of fasting in both rats and mice in spite of the large variations in the blood sugar concentra-

TABLE 1. EFFECT OF FASTING ON THE HYPOGLYCEMIC RESPONSE OF MICE TO EXOGENOUS INSULIN

0.0025 unit insulin in 0.02 ml. acidified water per gram body weight was administered intraperitoneally. All values expressed as Mean ±SE.

Fasting hours	No. of mice	Blood sugar—mg. per cent			Mean decrease per cent
		0	1 hour	2 hours	
0	10	129.5± 6.7	110.0±10.8	127.5±13.2	8.3
24	10	80.6± 5.7	58.4± 3.3	71.1± 5.3	19.6
40	11	90.6± 8.5	47.6± 6.9	59.7±10.0	44.8
72	11	100.6± 9.2	57.0±11.8	50.3±11.9	46.6
96	10	69.2± 4.6	40.0± 9.1	28.3± 6.1	50.6
114	10	67.0±15.4	38.2±16.1	24.8± 8.3	53.0

TABLE 2. EFFECT OF FASTING ON THE HYPOGLYCEMIC RESPONSE OF RATS TO EXOGENOUS INSULIN

0.05 unit insulin in 0.2 ml. acidified water per 100 gm. body weight was administered intraperitoneally. All values expressed as Mean ± S.E.

Fasting hours	No. of rats	Blood Sugar—mg. per cent					Mean decrease per cent
		0	1	2	3	4	
0	20	103.4±3.0	81.9±3.7	82.2±5.6	89.9±5.2	100.1±4.0	14.4
24	20	79.3±1.8	55.9±2.9	53.2±3.9	59.3±3.7	69.7±3.3	22.7
48	20	79.8±3.1	62.3±2.9	61.8±3.6	72.4±3.0	78.8±3.3	13.8
72	20	86.8±3.0	58.6±3.2	58.0±3.8	70.3±3.4	79.8±4.3	23.1
96	18	86.3±4.0	59.3±3.9	60.6±4.4	69.8±4.6	82.1±5.3	21.3
120	21	80.6±3.5	49.3±2.8	49.7±3.7	58.9±3.8	62.0±6.2	33.8
144	25	77.3±2.9	39.9±3.2	35.6±3.4	37.2±4.8	44.0±4.9	49.3

tion of the mice. Thereafter, the blood sugar concentration remains fairly constant in rats until the end of the period of fasting. In the mice, however, a statistically significant decrease ($p < 0.001$) occurs after the fourth day of fasting.

The "mean per cent decrease" in the blood sugar concentration of the rats, computed from the values for the hourly intervals after the injection of the insulin, serves as an index of the over-all hypoglycemic response (6). According to this criterion, rats exhibit an increased hypoglycemic response to a constant dose of insulin by the end of the first day of fasting, the mean per cent decrease in the blood sugar changing from 14.4 to 22.7. By the end

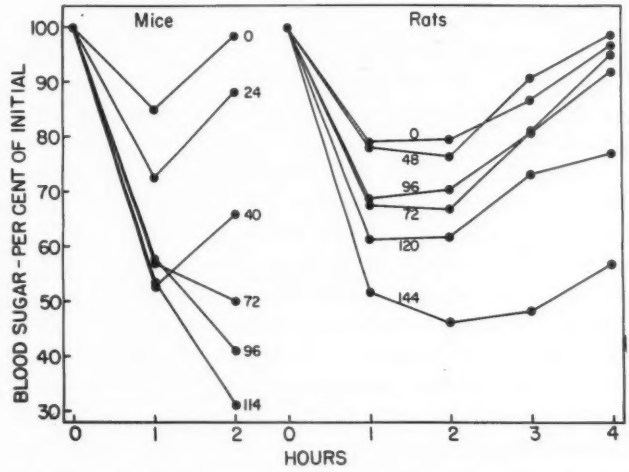

FIG. 2. The Effect of Fasting on the Hypoglycemic Response to Exogenous Insulin. The mean blood sugar concentration for each interval after the administration of insulin is expressed as the per cent of the initial concentration. Each curve represents the data obtained on a group of animals fasted for the stated period.

of the second day of the fast, the mean per cent decrease is back to 13.8. After three days of fasting, the over-all hypoglycemic response is similar to that observed by the end of the first day, viz., a mean decrease of 23.1%. No further significant change occurs in the hypoglycemic response until the end of five days of fasting when the mean per cent decrease in blood sugar is 33.8. It is pertinent to note that essentially there is a progressive increase in the immediate hypoglycemic response to insulin (i.e., during the first two hours) throughout the period of fasting and that the over-all hypoglycemic response during the first five days of fasting is determined principally by the rate of restitution of the blood sugar (i.e., during the third and fourth hours) which is slower after the first 24 hours of fasting and then remains at a relatively high level until the fifth day of fasting (Fig. 2).

In spite of the marked variation in the initial blood sugar concentration, the effect of fasting on the hypoglycemic reponse of mice to the injection of insulin is very striking (Table 1, Fig. 2). Thus, after 40 hours of fasting there is a marked increase in the hypoglycemic response. As with the rats, the intensity of the over-all hypoglycemic response is determined not only by the degree of the initial hypoglycemia but also by the rate of restitution of the blood sugar (Fig. 1). Comparison of the mean per cent decrease in the blood sugar concentration (Table 1) with the rate of insulin destruction (Fig. 1) by mice reveals a fairly close inverse relationship between these measures.

DISCUSSION

The destruction of insulin *in vitro* (7) and *in vivo* (4) is dependent upon the action of an enzyme system, insulinase, which is relatively specific in catalyzing the hydrolysis of insulin (8). The observation that intact mice exhibit a decrease in the rate of destruction of insulin after a fast of more than 40 hours is in accord with previous observations of a significant decrease in the insulinase activity of homogenates of livers from rats fasted for more than 48 hours (1). The data, however, are not in accord with our observations on the insulinase activity of slices of livers from fasted rats (3) but no explanation of the discrepancy is available.

Selye (9) has observed that within 24 hours after the onset of fasting, rats develop an increased sensitivity to the hypoglycemic action of insulin. By the end of 48 hours of fasting, however, Selye found such animals to be quite resistant to the action of insulin and to remain so for at least another day. The data reported herein are in agreement with Selye's observation of an increased hypoglycemic response to insulin after one day of fasting and in contrast with our own experience in the past (1). The discrepancy between our own observations may be due to the fact that whereas in the former study the blood sugar concentration was measured for only 2 hours after the injection of insulin, it was followed for four hours in the present

study. On the basis of the data presented herein, the over-all hypoglycemic response after a 24 hour period of fasting is dependent in large measure on the rate of restitution of the blood sugar and the latter will be missed if the study is confined to the first phase of the response to insulin.

In contrast with Selye's observations, no decrease in the hypoglycemic response to insulin was observed after 72 hours of fasting. In fact, the data reported herein reveal that fasting beyond 2 days enhances the hypoglycemic response to a standard dose of insulin in both rats and mice. The discrepancy between Selye's observations and the above may be due to differences in the quantity of insulin that was given and in the length of time that the animals were studied after the administration of the insulin.

The increased hypoglycemic response to exogenous insulin exhibited by the fasted rat and the fasted mouse can be accounted for by the decrease in the insulinase activity of such animals. A decrease in the rate of which the exogenous insulin is destroyed will make a larger proportion of the standard dose available for physiological action. Thus, the insulinase activity of homogenates of livers from rats fasted for 72 or more hours is markedly reduced while the hypoglycemic response to insulin is markedly enchanced after a similar period of fasting. Likewise, the insulinase activity of intact mice is markedly reduced after a fast of more than 42 hours while the hypoglycemic response to insulin is enhanced in mice fasted longer than 48 hours. The inverse relationship between the level of insulinase activity and the hypoglycemic response to exogenous insulin reveals the physiological significance of insulinase.

CONCLUSIONS

Fasting results in a marked reduction in the insulinase activity of mice and an enchancement of the hypoglycemic response of both mice and rats to exogenous insulin. The inverse relationship between the rate of insulin destruction and the hypoglycemic response to insulin suggests that insulinase is of physiological significance in the intact animal.

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BIOASSAY OF HYPOPHYSEAL GROWTH HORMONE IN HYPOPHYSECTOMIZED MICE BY THE TIBIA TEST[1]

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THE intact mouse has been found to be quite unresponsive to exogenously administered growth hormone (1, 2). No data, however, have heretofore been published on the effects of growth hormone in the hypophysectomized mouse; in the studies reported here, the responsiveness of hypophysectomized female mice of the C3H, A/He, BALB/c, and C57 BL strains has been assessed on the basis of increases in the width of the proximal epiphyseal cartilage of the tibia and of increases in body weight. Wherever possible, comparisons have been made between the results obtained in the hypophysectomized mouse and those reported for the hypophysectomized rat.

EXPERIMENTAL PROCEDURE

Female mice of the A/He, BALB/c, C3H and C57BL strains were hypophysectomized at 35 days of age according to the technic described in an earlier publication (3). Following the operation, 1 mg. of terramycin dissolved in 1 ml. of 5% glucose solution was given intraperitoneally. A similar injection was administered on the following day along with a subcutaneous injection of 0.25 mg. of hydrocortisone. Throughout the study, the animals were fed a modified McCollum's Diet I, *ad libitum* (4).

Growth hormone administration was begun 12 days later and was continued for a period of either 4 days or 17 days. The hormone solution was prepared every fifth day and, unless otherwise indicated, all injections were made subcutaneously once daily. At autopsy, the individual organs were weighed on a Roller-Smith torsion balance; the width of the proximal epiphyseal cartilage of the tibia was ascertained by the simplified procedure routinely used in this laboratory (5), which is a modification of the method originally described for the rat (6).

The growth hormone used in this study was prepared from bovine pituitaries according to the procedure previously described (7). Assays at a dose level of 10 mg. indicated that this hormone preparation contained no detectable FSH, ICSH, lactogenic hormone (prolactin) or ACTH, and less than 1% TSH[2].

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[2] The following abbreviations are used throughout the text: FSH, follicle-stimulating hormone; ICSH, interstitial cell-stimulating hormone; ACTH, adrenocorticotropic hormone; TSH, thyrotropic hormone.

RESULTS[3]

1. *The responses of four inbred strains to growth hormone.* Hypophysectomized female mice of the four inbred strains tested manifested a response to the exogeneously administered growth hormone. Relatively small amounts were effective, and near maximal increases in the width of the tibial epiphyseal cartilage were obtained with a daily dose of 0.015 mg. The average tibial widths are shown in Table 1. With one-tenth mg. of growth hormone per day weight gains of 5, 6½, 3 and 5 grams were obtained. The

TABLE 1. EFFECTS OF PITUITARY GROWTH HORMONE ON HYPOPHYSECTOMIZED FEMALE MICE

Strain	Operative condition*	Daily dose**	No. of animals	Body weights			Organ weights			Width of tibial epiphyseal cartilage plate
				Day of operation	Day of 1st inj.	At autopsy	Adrenal	Ovary	Uterus	
		mg.		gm.	gm.	gm.	mg.	mg.	mg.	micra
BALB/c	N	0	10	15	16	18½	5.4±0.2†	5.1±0.4	45.7± 9.3	103.5±2.2
	H	0	8	16	12	12½	2.1±0.2	1.1±0.1	5.9± 0.6	71.2±1.5
	H	0.015	5	18	14	18	—	—	—	138.9±2.7
	H	0.040	10	16	11	16½	2.2±0.1	1.5±0.1	6.0± 0.2	141.5±5.3
	H	0.100	8	16	11½	16½	2.4±0.2	1.5±0.2	5.6± 0.4	145.4±6.0
C3H	N	0	7	18	20	23	4.0±0.2	10.1±0.2	85.1± 9.1	119.8±2.8
	H	0	10	18	12½	14½	1.6±0.2	1.2±0.1	6.1± 0.6	58.0±1.1
	H	0.015	5	18	13½	17	—	—	—	136.9±3.0
	H	0.040	10	19½	13½	18½	2.1±0.14	1.5±0.1	8.0± 0.6	144.6±2.5
	H	0.100	10	18	12½	19	2.2±0.2	1.6±0.2	7.8± 0.8	159.7±3.2
A/He	N	0	11	15	17	19½	3.9±0.2	4.7±0.3	51.5±28.4	120.8±5.9
	H	0	10	16	12½	12½	1.5±0.1	1.1±0.1	5.3± 0.7	60.2±1.1
	H	0.015	4	16	12	14	—	—	—	135.9±4.1
	H	0.040	9	16	12	16	1.9±0.1	1.2±0.1	7.1± 0.5	123.4±1.1
	H	0.100	10	16	13	16	1.8±0.1	1.1±0.1	7.2± 0.5	146.0±3.1
C57BL	N	0	10	13	16	19	3.9±0.2	6.4±0.3	65.5± 3.2	119.2±1.7
	H	0	8	15	12½	13½	1.5±0.1	1.1±0.1	4.9± 0.5	48.5±2.3
	H	0.015	5	15	12	15	—	—	—	117.8±2.6
	H	0.040	8	15	12½	16½	1.9±0.1	1.2±0.1	6.4± 0.5	111.0±4.6
	H	0.100	8	15	12	17	2.0±0.2	1.7±0.1	6.1± 0.2	127.9±3.9

* N = Non-operated. H = Hypophysectomized. Animals operated on at 35 days-of-age and sacrificed at 65 days.
** Injections begun at 47 days-of-age and continued for 17 days.
† Mean ± standard error.

weights of the adrenal glands, ovaries and uteri of the hormone-injected groups did not differ significantly from those of the controls.

Further studies with the C3H strain revealed that the increase in the width of the epiphyseal cartilage plate of the tibia of the hypophysectomized mouse is both a sensitive index for and a quantitative measure of exogenous growth hormone activity. For example, less than 0.001 mg. per day is effective in the seventeen-day tibia test. These results are summarized in Table 2 and are plotted in Figure 1 in the form of a log dose-response curve, which was obtained by the method of least squares and can be expressed by the following equation:

$$Y = 84.3 + 47.2 \log x \qquad (1)$$

where Y equals the response of the cartilage plate in micra and x equals the daily dose of growth hormone in micrograms. The calculated index

[3] The authors wish to acknowledge the able technical assistance of Charles W. Jordan.

TABLE 2. THE TIBIAL RESPONSE OF THE HYPOPHYSECTOMIZED* FEMALE C3H MOUSE TO PITUITARY GROWTH HORMONE

Daily dose**	No. of animals	Body Weights			Width of tibial epiphyseal cartilage plate
		Day of operation	Day of 1st inj.	At autopsy	
mg.		gm.	gm.	gm.	micra
0	10	18	12½	14½	58.0±1.1†
0.001	15	15½	12	14	85.6±2.1
0.002	6	16	12½	14	95.8±1.5
0.0035	5	17½	13	15	110.9±1.9
0.005	15	15	12	15	120.1±1.1
0.007	6	16½	13	15½	121.4±0.9
0.010	5	16	12	15½	131.0±2.6
0.020	13	15½	12½	16	147.0±2.5

* All animals hypophysectomized at 35 days-of-age and sacrificed at 65 days.
** Growth hormone injections begun at 47 days-of-age and continued for 17 days.
† Mean ± standard error.

of precision, λ, is 0.17. Photographs of tibiae from control and growth hormone-treated animals are shown in Figure 2.

2. *The effect of alterations in the injection procedure.* The response of the hypophysectomized mouse to exogenous growth hormone is influenced by the route and by the frequency of injection. A dose of 0.010 mg. administered intraperitoneally for seventeen days produced an average tibial width of 100.6 micra. Subcutaneous injections given once daily yielded an average value of 119.3 micra, whereas subcutaneous injections given twice daily gave an average measurement of 130.9 micra (Table 3).

3. *The influence of other hormones on the tibial response.* While a significant change in the width of the cartilage plate could be obtained with a daily dose of 0.001 mg. of growth hormone given over a period of 17 days, ten times this level was required to produce a comparable response within a 4-day period. This limited tibial response does not represent the maximal stimulation that can be obtained with growth hormone alone during the 4-day period. As can be seen in Table 4, 0.50 mg. per day elicited an increase equivalent to the dose of 0.033 mg. given for the seventeen-day period, as computed from Equation (1).

These data suggest that the hypophysectomized mouse is deficient in

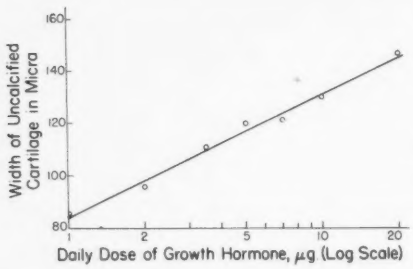

FIG. 1. Effect of hypophyseal growth hormone on the width of the proximal epiphyseal cartilage of the tibia of the hypophysectomized mouse.

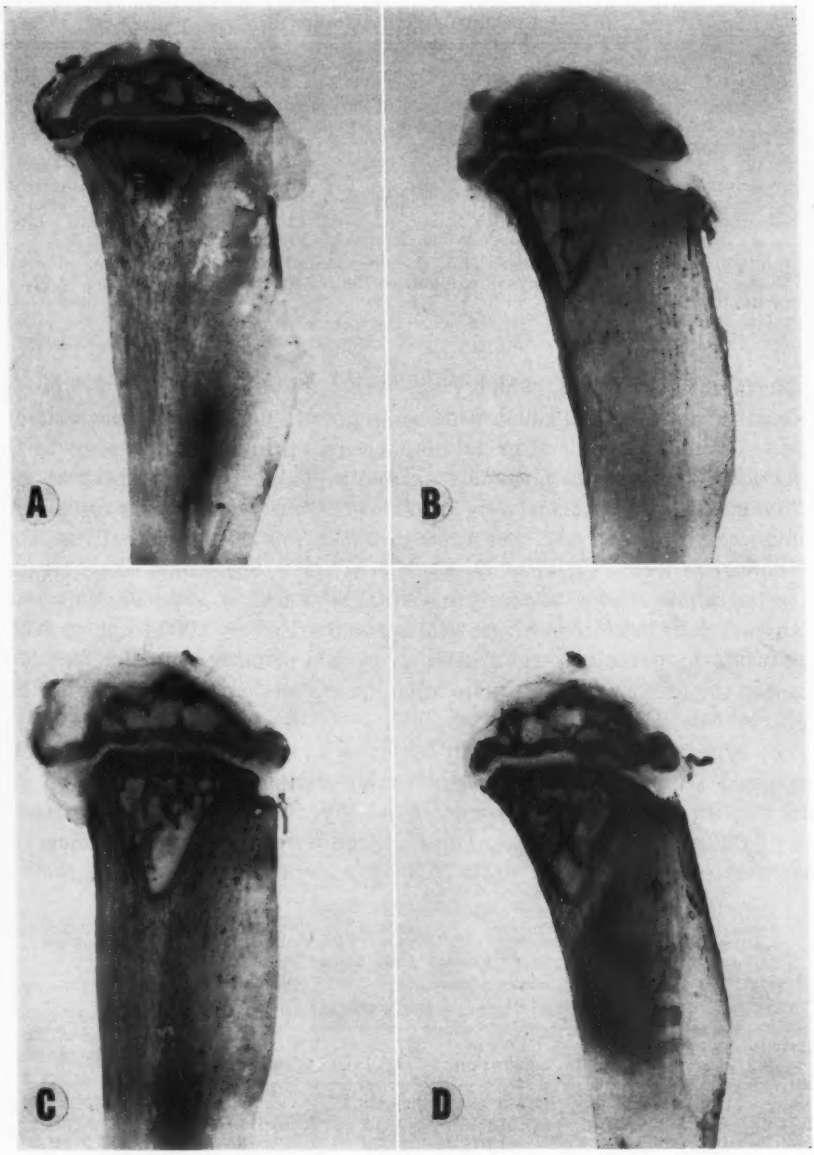

FIG. 2. Effect of increasing doses of growth hormone on the proximal epiphyseal cartilage of the tibia of the hypophysectomized mouse in 17 days. (A) Control; (B) 1 microgram; (C) 5 micrograms; (D) 20 micrograms.

TABLE 3. THE INFLUENCE OF THE ROUTE AND OF THE FREQUENCY OF INJECTION ON THE TIBIAL RESPONSE OF THE HYPOPHYSECTOMIZED* FEMALE C3H MOUSE TO GROWTH HORMONE**

Route	Frequency of daily injection	No. of animals	Body weights			Width of tibial epiphyseal cartilage plate
			Day of operation	Day of 1st. inj.	At autopsy	
			gm.	gm.	gm.	micra
Intraperitoneal	Once	7	16	12	14½	100.6±5.6†
Subcutaneous	Once	10	16	12	15	119.3±1.7
Subcutaneous	Twice	10	15	12	15	130.9±1.6

* All animals hypophysectomized at 35 days-of-age and sacrificed at 65 days.
** Injections begun at 47 days-of-age and continued for 17 days. Daily dose of growth hormone, 0.005 mg.
† Mean ± standard error.

some factor (or factors) which plays either a synergistic or a permissive role with respect to the tibial response to growth hormone. Hydrocortisone and thyroxine were therefore administered as priming agents prior to the injection of the growth hormone. As seen in Table 5, animals primed with 0.25 mg. of hydrocortisone were no more responsive than their nonprimed litter mates; in fact, the measured response was actually less than that obtained with growth hormone alone. On the other hand, those treated with thyroxine showed a nearly maximal response to a specific dose level with just four injections of growth hormone. Either 0.0003 mg. or 0.002 mg. of DL-thyroxine given concurrently or as a priming agent for five days prior to the first growth hormone injection effected an enhancement of the response (see Tables 6 and 7).

The synergism between growth hormone and thyroxine with respect to the tibial stimulation that was observed during the 4-day period was striking; for example, 0.0001 mg. of DL-thyroxine injected concurrently with 0.005 mg. of growth hormone elicited a response of 91.1 micra, in contrast to a response of only 76.3 to growth hormone alone. Such a

TABLE 4. THE INFLUENCE OF THE INJECTION PERIOD ON THE TIBIAL RESPONSE OF THE HYPOPHYSECTOMIZED FEMALE C3H MOUSE* TO GROWTH HORMONE

Injection period	Daily dose**	No. of animals	Body weights			Width of tibial epiphyseal cartilage plate
			Day of operation	Day of 1st inj.	At autopsy	
days	mg.		gm.	gm.	gm.	
4	0	12	13½	11	11½	57.8±0.8†
4	0.010	8	15	12	13	87.2±3.0
4	0.500	7	14	12	13½	156.1±3.6
17	0	10	18	12½	14½	58.0±1.1
17	0.001	15	15½	12	14	85.6±2.1
17	0.010	5	16	12	15½	131.0±2.6

* All animals hypophysectomized at 35 days-of-age.
** All injections begun at 47 days-of-age and continued for interval indicated.
† Mean ± standard error.

TABLE 5. EFFECT OF A PRIMING DOSE* OF HYDROCORTISONE ON THE TIBIAL RESPONSE OF THE HYPOPHYSECTOMIZED FEMALE C3H MOUSE TO GROWTH HORMONE**

Preparation	Injection interval	No. of animals	Body weights			Width of tibial epiphyseal cartilage plate
			Day of operation	Day of 1st inj.	At autopsy	
	days		gm.	gm.	gm.	micra
Saline	4	12	13½	11	11½	57.8±0.8†
Saline+hydrocortisone	4	6	15½	11	11	62.5±0.8
GH	4	5	14½	11	12½	93.3±4.8
GH+hydrocortisone	4	9	15	11½	12	70.3±2.7
Saline	17	10	18	12½	14½	58.0±1.1
Saline+hydrocortisone	17	5	18	13½	14½	53.5±2.4
GH	17	5	18	13½	17	136.9±3.0
GH+hydrocortisone	17	9	15	11½	14½	121.8±2.6

* Single priming dose, 0.25 mg., given subcutaneously 5 days prior to first growth hormone injection.

** Daily dose of growth hormone (GH), 0.015 mg. All animals hypophysectomized at 35 days-of-age. Injections of growth hormone begun at 47 days-of-age.

† Mean ±standard error.

synergism was not observed in the 17-day period; the combination of the two hormones administered at those same dose levels for the longer period produced no enhancement of the measured response. However, 0.0003 mg. of DL-thyroxine injected concurrently with 0.001 mg. of growth hormone increased the width of the cartilage plate from 85.6 micra to 107.4 micra, suggesting that a synergism between the two hormones can be demonstrated over longer injection periods if proper dose levels are employed.

Thyroxine not only acted as a synergist to the growth hormone when administered concurrently with the latter during the 4-day period, but also was itself active in the tibia test. Mice given 0.0003 mg. of DL-thyroxine registered an average cartilage plate width of 66.6 micra; that of the

TABLE 6. EFFECT OF PRIMING TREATMENT WITH THYROXINE* ON THE TIBIAL RESPONSE OF THE HYPOPHYSECTOMIZED** FEMALE C3H MOUSE TO GROWTH HORMONE IN 4 DAYS

Preparation†	Daily dose	No. of animals	Body weights			Width of tibial epiphyseal cartilage plate
			Day of operation	Day of 1st inj.	At autopsy	
	mg.		gm.	gm.	gm.	micra
Saline	0	12	13½	11	11½	57.8±0.8††
Tx	0.0003	10	15	12	12	59.8±1.0
Tx	0.002	13	15½	12	13	81.6±2.0
GH	0.010	8	15	12	13	87.2±3.0
GH+Tx	0.010+0.0003	7	15½	12	13	96.0±3.7
GH+Tx	0.010+0.002	13	16	12	13	117.4±1.7

* Priming dose of DL-thyroxine (Squibb) given daily, subcutaneously, for 5 days preceding first growth hormone injection.

** All animals hypophysectomized at 35 days and sacrificed at 52 days.

† Tx, DL-thyroxine; GH, growth hormone. Injections begun at 47 days-of-age and continued for 4 days.

†† Mean ±standard error.

TABLE 7. EFFECT OF THYROXINE EITHER ALONE OR IN COMBINATION WITH GROWTH HORMONE ON THE TIBIAL RESPONSE OF THE HYPOPHYSECTOMIZED* C3H MOUSE IN 4 DAYS

Preparation**	Daily dose	No. of animals	Body weights			Wifth of tibial epiphyseal cartilage plate
			Day of operation	Day of 1st. inj.	At autopsy	
	mg.		gm.	gm.	gm.	micra
Saline	0	12	13½	11	11½	57.8±0.8†
Tx	0.0001	8	15½	11½	11½	60.9±1.2
Tx	0.0003	16	15½	12½	12½	66.6±1.0
GH	0.005	6	15½	12	12½	76.3±4.6
GH	0.010	8	15	12	13	87.2±3.0
GH+Tx	0.001+0.0001	6	14½	11	11	65.0±3.4
GH+Tx	0.005+0.0001	5	15½	12	13	91.1±6.5
GH+Tx	0.005+0.0003	7	15½	12	12½	94.6±3.1
GH+Tx	0.010+0.0003	11	15½	12	13	120.6±2.2

* All animals hypophysectomized at 35 days-of-age and sacrificed at 52 days.
** Tx, DL-thyroxine (Squibb), GH, growth hormone. Injections begun at 47 days-of-age and continued for 4 days.
† Mean ±standard error.

controls was 57.8 micra. When this same dose of thyroxine was supplied for 17 days, the cartilage plate width was increased to 75.5 micra, while animals injected with this level of thyroxine for the entire 30 day period exhibited a tibial response of 93.3 micra. These data are summarized in Tables 7 and 8.

DISCUSSION

The results obtained with growth hormone in the four inbred strains of mice are more striking for their similarities than for their differences. Although the 17-day regimen of injections was designed to accentuate strain differences, entirely comparable results were obtained in the C3H,

TABLE 8. EFFECT OF THYROXINE EITHER ALONE OR IN COMBINATION WITH GROWTH HORMONE ON THE TIBIAL RESPONSE OF THE HYPOPHYSECTOMIZED* C3H MOUSE IN 17 DAYS

Preparation**	Daily dose	No. of animals	Body weights			Width of tibial epiphyseal cartilage plate
			Day of operation	Day of 1st inj.	At autopsy	
	mg.		gm.	gm.	gm.	micra
Saline	0	10	18	12½	14½	58.0±1.1†
Tx	0.0001	5	15½	11	11½	66.2±2.8
Tx	0.0003	10	16	12	13½	75.5±1.4
GH	0.001	15	15½	12	14	85.6±2.1
GH	0.0035	5	17½	13	15	110.9±1.9
GH	0.005	15	15	12	15	120.1±1.1
GH	0.010	5	16	12	15½	131.0±2.6
GH+Tx	0.0035+0.0001	7	16	13	16	106.9±1.2
GH+Tx	0.005+0.0001	5	15½	12½	15½	114.5±2.7
GH+Tx	0.001+0.0003	6	15½	12	15	107.4±3.5

* All animals hypophysectomized at 35 days-of-age and sacrificed at 65 days.
** Tx, DL-thyroxine (Squibb); GH, growth hormone. Injections begun at 47 days-of-age and continued for 17 days.
† Mean ±standard error.

A/He and BALB/c strains given 0.015 mg. of the same hormonal solution. Inasmuch as untreated hypophysectomized A/He mice invariably succumb a short time after the operation (3), both the growth hormone and the saline-injected groups were given 0.25 mg. of hydrocortisone every tenth day. With the hydrocortisone treatment, the A/He strain exhibited a tibial response entirely comparable to that of the C3H and BALB/c. On the other hand, the C57BL mice showed a lower but maximal stimulation with 0.015 mg. of the same growth hormone solution. It seems entirely possible that some additional factor, for example thyroxine, may be required by these animals in order for them to show further growth. This aspect of the problem was not explored.

The more extensive studies in the C3H mice demonstrate that the responses elicited in the hypophysectomized mouse with growth hormone are similar to those seen in the rat. Both species respond to relatively low dosages of the hormone, and the increase in the width of the proximal epiphyseal cartilage of the tibia provides a quantitative measure of the hormonal activity. Equation (1) indicates that the slope in the mouse assay is not as steep as that recently reported for the rat (5). However, the deviations of the individual values from the mean response are small, and the excellent value of 0.17 for the index of precision, λ, attests to the accuracy of the method. Moreover, the absolute values for the cartilage widths are remarkably reproducible in groups of mice tested at different times.

There are specific differences between tibial responses observed in the hypophysectomized C3H mouse and those observed in the hypophysectomized Long-Evans rat. While four daily injections of growth hormone produce a maximal increase in the epiphyseal cartilage of the rat (8), only a limited increase is obtained in the mouse, the order of sensitivity being approximately one-tenth that of the rat. In the mouse, injections for seventeen days do produce results comparable to those obtained in the rat with four injections. Studies were therefore undertaken to ascertain whether treatment with either hydrocortisone or thyroxine would permit the hypophysectomized mouse to respond as fully to growth hormone in four days as it does in seventeen days.

Priming with hydrocortisone does not accelerate the process, but actually antagonizes the growth hormone effect. A similar inhibition has been demonstrated in the rat with hydrocortisone and with ACTH (5). However, simultaneous treatment with 0.0003 mg. of DL-thyroxine does permit the hypophysectomized C3H mouse to respond approximately one-half as effectively in four days to a given dose of growth hormone as in seventeen days. Priming treatment with this same level of thyroxine is much less effective (Tables 6 and 7).

The insensitivity of the hypophysectomized mouse to growth hormone administered for only four days resembles that of the hypophysectomized-

thyroidectomized rat. In both cases, relatively large doses of growth hormone are required in order to effect tibial stimulation. Furthermore, the simultaneous administration of minute dosages of thyroxine appears to sensitize the animals and to restore partially their full capacity for responding to the injected growth hormone (9). In the hypophysectomized mouse, treatment with levels of thyroxine in either maintenance doses, priming doses, or doses administered simultaneously with growth hormone, did not permit the injected animals to exhibit responses in the 4-day period equivalent to those obtained with growth hormone alone in the seventeen-day period. No data were obtained for the hypophysectomized-thryoidectomized mouse, and the effects of growth hormone in the doubly-operated animal remain to be assessed.

It has been clearly demonstrated that microgram levels of thyroxine given to hypophysectomized rats elicit significant increases in the width of the epiphyseal cartilage plate (5, 9). A similar effect is seen in the mouse where increases in width accompany increasing dosages and where the effect of thyroxine is cumulative over extended injection periods. Thyroxine likewise increases the responsiveness of hypophysectomized rats to growth hormone. The enhancement is quantitative, and the slope of the dose-response curve in thyroxine-treated and non-treated rats is essentially the same for a given preparation of growth hormone (5). It remains to be determined whether or not the mouse will respond in a similar way. However, it is apparent that 0.0001 mg. of DL-thyroxine given in conjunction with 0.0035 mg. or with 0.005 mg. of growth hormone for seventeen days did not increase the measured tibial response above that obtained with the growth hormone alone, whereas 0.0003 mg. of thyroxine injected concurrently with 0.001 mg. of growth hormone effected some enhancement in the response.

Although neither the daily frequency nor the route of injection affects the tibia assay for growth hormone in the rat (8), both aspects must be considered in the mouse. Subcutaneous injections are more effective than intraperitoneal injections, and subcutaneous injections given twice daily are considerably more effective than those given intraperitoneally once daily. No explanation for the differences observed in the two species is apparent.

SUMMARY

Hypophysectomized female mice are quite sensitive to exogenously administered growth hormone. In the mouse, as in the rat, the increase in the width of the proximal epiphyseal cartilage of the tibia is a quantitative measure for growth hormone activity. Of the four inbred strains studied, the BALB/c, A/He and C3H strains showed comparable tibial stimulation when injected once daily for seventeen days with 0.015 mg. of growth

hormone; in the C57BL mice, a smaller, although maximal for that strain, increase was obtained.

Further studies in the C3H mouse showed that a significant tibial stimulation could be elicited with a daily dose level of 0.001 mg. of growth hormone injected for seventeen days; the response is proportional to the log dose that is administered, as represented by Equation (1). Subcutaneous injections given twice daily are more effective than those given once daily and are considerably more effective than single intraperitoneal injections.

In the 4-day test, the mouse was approximately one-tenth as sensitive as in the seventeen-day test; however, as little as 0.0003 mg. of DL-thyroxine enhanced the effect of growth hormone and permitted the mice to respond about one-half as effectively with four injections of a given dose of growth hormone as with seventeen. Furthermore, thyroxine not only acted as a synergist to growth hormone in the four-day test, but was also itself effective in increasing the width of the cartilage plate of the tibia.

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THE HYPOGLYCEMIC ACTION OF METABOLIC DERIVATIVES OF L-TRYPTOPHAN BY MOUTH[1,2]

I. ARTHUR MIRSKY, GLADYS PERISUTTI AND ROBERT JINKS *with the technical assistance of* MARY KAUFMAN

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THE administration of L-tryptophan by stomach tube results in a significant decrease in the blood sugar concentration of normal rats but not of severely diabetic alloxanized rats. Concomitant with the hypoglycemic response to L-tryptophan by mouth is a decrease in the insulinase activity of the intact normal rat and mouse (1, 2). Accordingly, the hypoglycemic response may be attributed in part at least to an increase in the availability of endogenous insulin consequent to a decrease in the rate of insulin destruction.

In view of the hypoglycemic action of L-tryptophan, it became pertinent to study the action of various metabolic products of the amino acid on the blood sugar of normal and diabetic rats.

METHOD

The L-tryptophan derivatives were dissolved or suspended in 0.5% sodium bicarbonate and adjusted to pH 8.0. The concentration of the solution was such as to permit the administration of from 0.1 mM to 0.4 mM of the compound in 5 ml. per 100 gm. of body weight by stomach tube. Each compound was given to 10 or more male rats of the Carworth strain, weighing from 150 to 300 gm. after an overnight fast. Blood samples were taken from the clipped tail into 0.1 ml. pipettes immediately before and at hourly intervals for five hours after the gavage. The glucose concentration was determined by Nelson's procedure (3). In those instances where hydroxylated derivatives of L-tryptophan was given, the blood glucose concentration was estimated from the difference between fermented and non-fermented fractions of blood filtrates (4).

Alloxan diabetes mellitus was produced in rats by the subcutaneous injection of 180 mg. alloxan (5% solution) per kilogram of body weight. Forty-eight hours thereafter, those animals which developed a significant glycosuria were started on a maintenance dose of 1 unit protamine zinc insulin[3] per day. Approximately one month later, the insulin was discontinued for two days and those rats which did not show an appreciable decrease in the blood sugar concentration after an overnight fast were used in this study.

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[3] We are indebted to Dr. W. R. Kirtley of the Eli Lilly Company for generous supplies of insulin.

RESULTS

The data on the effect of L-tryptophan and of some of its metabolic products on the blood sugar concentration of rats are summarized in Table 1. Some of these data are depicted in Figure 1 and Figure 2 where the blood sugar concentration at each interval after the gavage is expressed as the per cent of the initial concentration. It is apparent that whereas the administration of a solution of sodium bicarbonate results in a slight increase in the blood sugar by the end of the fifth hour, a statistically significant hypoglycemic response is produced by the administration of dl-

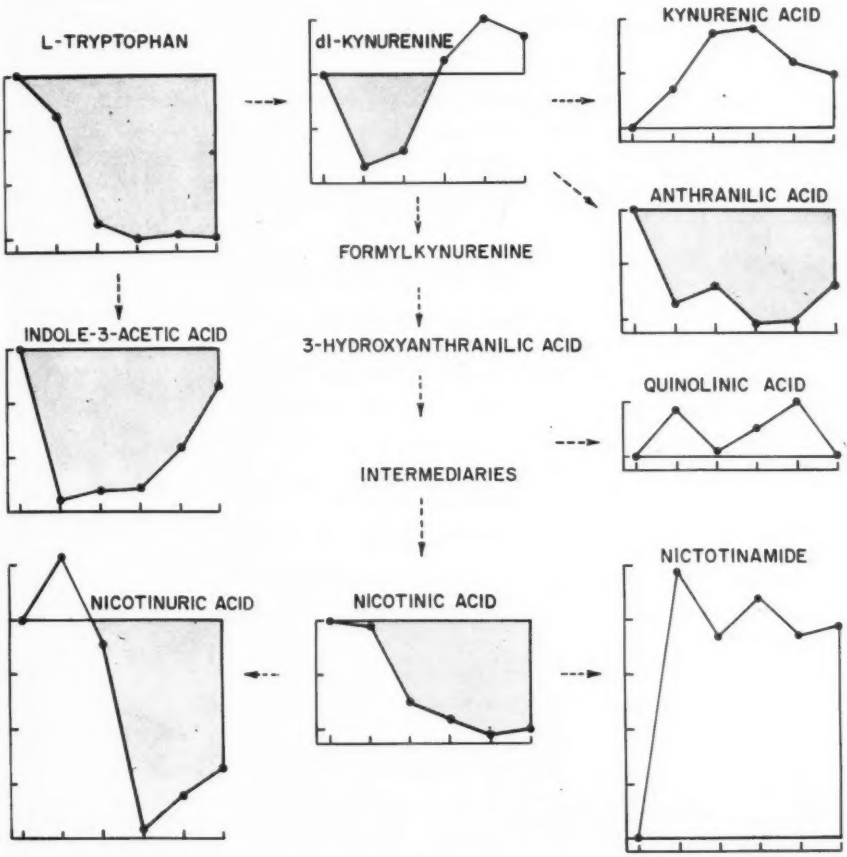

FIG. 1. Effect of Metabolic Derivatives of L-Tryptophan on the Blood Sugar of Rats.

The mean blood sugar concentration at each hourly interval after the administration of 0.4 mM per 100 grams per os is expressed as the per cent of the initial concentration. Each division of the ordinate equals 10 per cent and each division of the abscissa equals 1 hour. A decrease from the initial blood sugar concentration is depicted as a shaded area.

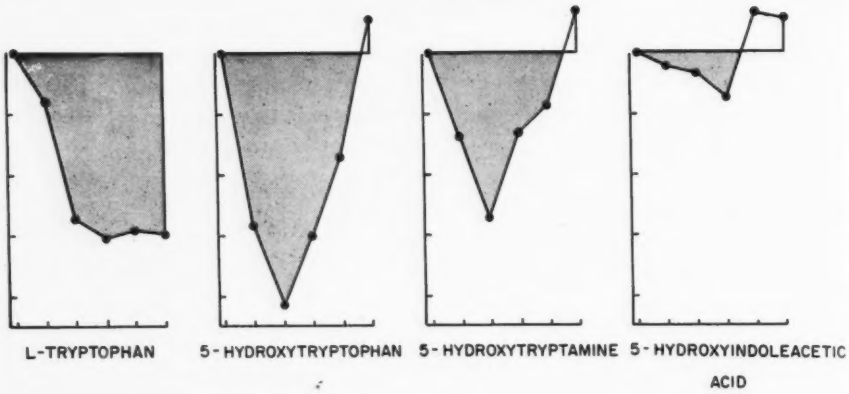

FIG. 2. Effect of Hydroxylated Products of L-Tryptophan on the Blood Sugar of Rats.

The mean blood sugar concentration at each hourly interval after the administration of 0.4 mM per 100 grams per os is expressed as the per cent of the initial concentration. Each division of the ordinate equals 10 per cent and each division of the abscissa equals 1 hour. A decrease from the initial blood sugar concentration is depicted as a shaded area.

kynurenine, anthranilic acid, nicotinic acid, nicotinuric acid, indole-3-acetic acid, 5-hydroxytryptophan and 5-hydroxytryptamine creatinine sulfate. The oral administration of nicotinamide and kynurenic acid results in a significant hyperglycemic response. The response of the blood sugar to the gavage with indole, skatole, quinolinic acid, picolinic acid and 5-hydroxyindoleacetic acid is essentially the same as to the sodium bicarbonate solution in which these compounds were dissolved.

TABLE 1. THE EFFECT OF METABOLIC PRODUCTS OF L-TRYPTOPHAN ON BLOOD SUGAR OF RATS

The various compounds were administered by stomach tube in a dosage of 0.4 mM per 100 grams of body weight.

Compound	No.	Blood sugar—mgm. per cent*						Mean‡ change per cent
		0	1 hour	2 hours	3 hours	4 hours	5 hours	
Sodium Bicarbonate	20	75.6±2.7	76.9±2.6	75.5± 2.3	81.2±1.8	79.8± 2.4	86.5± 3.6	+ 5.8
L-Tryptophan	34	89.6±3.0	85.5±3.1	66.1± 2.0	65.2±2.5	66.9± 5.1	70.2± 4.2	−21.0
5-Hydroxytryptophan	10	88.8±4.1	63.8±8.5	52.1±12.4	62.1±9.2	74.0±15.3	93.8±10.8	−22.1
Indole-3-acetic Acid	25	68.3±2.5	49.0±1.7	50.7± 1.8	52.8±1.7	56.2± 2.3	63.5± 3.6	−20.3
Nicotinuric Acid	10	93.2±4.4	103.9±5.8	89.0± 6.7	57.4±6.4	63.1± 8.4	67.5± 4.9	−18.3
Anthranilic Acid	10	76.7±4.8	63.3±5.5	66.4± 2.3	60.5±2.8	61.0± 2.5	66.0± 3.7	−17.3
Nicotinic Acid	25	80.4±2.5	79.6±3.8	68.5± 3.1	65.9±3.2	63.4± 1.9	64.4± 1.4	−15.0
5-Hydroxytryptamine	10	86.1±3.7	65.8±7.0	54.3± 4.8	75.0±6.4	78.8± 6.9	92.1± 5.5	−15.0
dl-Kynurenine	15	70.7±2.1	58.7±2.5	61.0± 3.7	72.4±2.9	77.9± 2.6	76.7± 2.0	−1.9
Picolinic Acid†	10	75.9±3.6	71.9±3.1	64.8± 2.7	71.4±2.7	84.4± 5.1	83.3± 3.0	−0.9
5-Hydroxy-Indoleacetic Acid	10	82.6±3.0	80.6±3.3	79.9± 4.2	76.3±3.8	88.0± 3.0	87.4± 2.7	−0.2
Quinolinic Acid	10	72.8±2.6	78.9±4.6	73.6± 2.9	76.2±3.4	80.8± 3.9	69.9± 3.0	+4.1
Skatole	10	77.6±4.7	84.0±5.2	73.6± 5.2	81.2±2.0	83.0± 4.0	82.8± 4.2	+4.3
Indole	10	69.7±2.9	83.6±4.5	82.1± 5.1	74.4±4.6	74.8± 3.4	71.2± 4.1	+10.8
Kynurenic Acid	11	69.1±2.4	73.8±2.5	81.0± 2.7	81.5±3.1	77.3± 2.3	76.3± 1.9	+11.3
Nicotinamide	15	62.9±2.6	92.9±2.5	85.9± 3.2	90.5±2.7	85.9± 1.3	87.2± 2.5	+40.7

* Mean ± S.E.
† 3 mM/k.
‡ Mean change computed from the five hourly blood samples and expressed as per cent of the initial concentration.

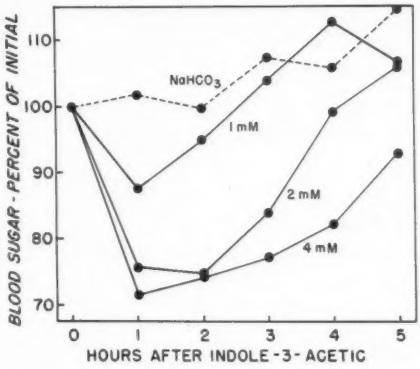

FIG. 3. Effect of Various Concentrations of Indole-3-Acetic Acid on the Blood Sugar of Rats.

Each curve represents the mean response of the blood sugar of from 10 to 20 rats. The blood sugar concentration at each interval is expressed as the per cent of the initial concentration. The dosage of the indole-3-acetic acid expressed as mM per kilo.

Whereas the hypoglycemic response becomes significant in about two hours after the administration of L-tryptophan, the maximum hypoglycemic response to nicotinic acid and nicotinuric acid is attained later while that to indole-3-acetic acid is apparent earlier (Fig. 1). As is illustrated with indole-3-acetic acid (Fig. 3), smaller concentrations of the hypoglycemic compounds produce a smaller but definite decrease in the blood sugar concentration.

In order to determine whether the hypoglycemic response to some of the metabolic products of L-tryptophan is dependent upon the availability of endogenous insulin, indole-3-acetic acid and nicotinic acid were administered to groups of from 10 to 15 severely diabetic alloxanized rats after an overnight fast. It is apparent from Figure 4 that the administration of indole-3-acetic acid does not decrease the blood sugar concentration of the diabetic rats whereas the same dose (0.4 mM per 100 gm.) is effective in non-diabetic animals. Similar results were obtained with nicotinic acid.

DISCUSSION

There are four major pathways for the degradation of L-tryptophan (5). One pathway, which is dependent upon the oxidative cleavage of the indole

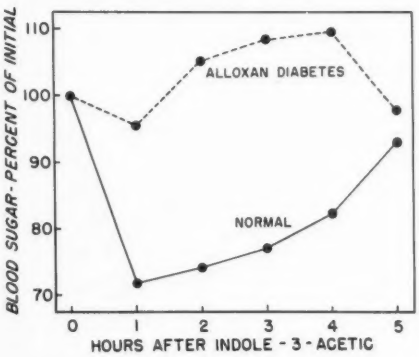

FIG. 4. Effect of Indole-3-Acetic Acid on the Blood Sugar of Alloxanized Diabetic Rats.

Each curve represents the mean response of the blood sugar of from 10 to 20 rats. The blood sugar concentration at each interval is expressed as the per cent of the initial concentration (316 ± 16.4 mg. per cent). The dosage of the indole-3-acetic acid expressed as mM per kilo.

ring, leads to the production of formylkynurenine, kynurenine, kynurenic acid, anthranilic acid, quinolinic acid, nicotinic acid, nicotinamide and nicotinuric acid among many other products (Fig. 1). In the alloxan diabetic animal, picolinic acid is formed, presumably from the oxidation of 3-hydroxyanthranilic acid (6). A second pathway which is dependent upon the hydroxylation of the ring leads to the formation of 5-hydroxytryptophan, 5-hydroxytryptamine (serotonin, enteramine) and 5-hydroxyindoleacetic acid (Fig. 2). A third pathway is through modification of the side-chain and the formation of indolepyruvic and indole-3-acetic acids. The fourth pathway is the hydrolysis of the L-tryptophan to indole, pyruvate and ammonia. Since only a relatively small proportion of administered tryptophan can be accounted for by all these pathways, it is quite possible that others exist for the degradation of this amino acid (7).

With the exception of studies with nicotinic acid and nicotinamide, there is essentially no information available on the action of the various metabolic derivatives of L-tryptophan on the blood sugar concentration of rats or other species. The reports on the effect of nicotinic acid and nicotinamide on the blood sugar are both numerous and contradictory; there are reports that they increase the blood sugar (8–12), that they lower the blood sugar (13–22) and that they do not effect the blood sugar (23–27). The consensus, however, seems to be that some degree of hypoglycemia is produced by the enteral and parenteral administration of nicotinic acid and nicotinamide to man and other species. Correll *et al.* (28) found that the intravenous injection of 5-hydroxytryptamine creatinine sulfate rapidly produces a marked hyperglycemia in rats and rabbits. Zambotti and de Bernard (28) report that indole-3-acetic acid reduces the blood sugar and increases the liver glycogen of rats.

The data presented herein do not provide any clarification of the contradictory findings mentioned above. It is apparent, however, that the insulinase-inhibitory and hypoglycemic action of L-tryptophan (1) is not dependent upon the product of any specific pathway of degradation. Thus, the oral administration of kynurenine, anthranilic acid, nicotinic acid and nicotinuric acid, which are products of the oxidative cleavage of the indole ring, produce a significant decrease in the blood sugar of rats. Yet kynurenic acid and nicotinamide by mouth produce an increase in the blood sugar. Administration of 5-hydroxytryptophan and 5-hydroxytryptamine creatinine sulfate, products of the hydroxylation of tryptophan (Fig. 2), result in a reduction of the blood sugar, while 5-hydroxyindoleacetic acid is relatively ineffective. Indole-3-acetic acid, a product of the modification of the side-chain of tryptophan produces a significant hypoglycemic response. The hydrolytic products of L-tryptophan, indole and skatole, produce no significant change in the blood sugar when given by mouth to rats.

Besides their relation as degradative products of L-tryptophan, some of the compounds which are effective as hypoglycemic agents have in common an effect on the growth of plants. Indole-3-acetic acid is the major natural plant growth hormone (auxin) (30). 5-hydroxytryptamine appears to resemble indole-3-acetic acid in some of its effects on plants (31). 5-hydroxyindoleacetic acid exhibits a small degree of auxin activity (32). Likewise, anthranilic acid has been reported to exert an auxin-like influence on plants (33). The effect of nicotinic and nicotinuric acids on the growth of plants is such as to permit them to be categorized as plant growth hormones (34, 35). Although there is no information available concerning the plant growth regulatory activity of the other derivatives which produce hypoglycemia, they fulfill some of the known structural requirements for such activity (30). The possibility that the hypoglycemic action of the effective derivatives of tryptophan is related to their capacity to function as plant growth regulators led to studies on the influence of a variety of natural and synthetic plant growth regulators on the blood sugar of rats and the observation that a variety of such plant growth regulators are effective also as hypoglycemic agents when given by stomach tube to rats (36).

Irrespective of the structural characteristics that determine the activity of the metabolic derivatives of tryptophan, the hypoglycemia that is produced is dependent upon the presence of insulin. The fact that the severely diabetic alloxanized rat does not respond to the administration of indole-3-acetic acid or nicotinic acid indicates that the response in the normal rat cannot be due to either a decrease in the secretion or in the activity of some hyperglycemic factor or to an acute hepatotoxic action. However, the response in the normal but not the diabetic rat may be due to an increase in the rate of insulin secretion by the Islets of Langerhans or to a decrease in the rate of destruction of endogenous insulin consequent to an inhibition of insulinase. That the latter is more probable is indicated by the demonstration of insulinase inhibition by the tryptophan derivatives which exert a hypoglycemic effect (37).

CONCLUSIONS

The oral administration of L-tryptophan, dl-kynurenine, anthranilic acid, nicotinic acid, nicotinuric acid, indole-3-acetic acid, 5-hydroxytryptophan and 5-hydroxytryptamine creatinine sulfate produce a significant hypoglycemia in normal rats. Kynurenic acid produces a slight and nicotinamide a significant hyperglycemia. Quinolinic acid, picolinic acid, 5-hydroxyindoleacetic acid, indole and skatole by mouth produce no significant changes in the blood sugar. The administration of indole-3-acetic or nicotinic acids to severely diabetic alloxanized rats does not reduce the blood sugar.

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NOTES AND COMMENTS

THYROID FUNCTION IN FETAL SHEEP[1]

Daily administration of I^{131} to sheep was been shown to cause damage in newborn lambs when the doses given were sufficient to cause thyroid destruction in the dam. (1) In order to relate observed damage to the fetal thyroid concentration of I^{131} at various periods of gestation a series of studies was initiated. This paper reports the results of the first study concerning inception of thyroid function on the basis of histological development and the uptake of radioiodine by the fetal thyroid gland.

The time of beginning concentration of radioiodine by the fetal thyroid has been determined by many investigators (2–8) for man and several species of animals. Initial uptake of stable iodine in fetal sheep has been reported by Fenger between the third and fourth month of gestation (9) and by Hogben during the third month (10).

METHODS

Fetuses were removed by cesarean section from seven yearling Suffolk ewes 46, 48, 50, 52, 58, 60, and 70 days after breeding. Six of the ewes were fed a food pellet containing 135 μc. of I^{131} at 40 hours and again at 20 hours before surgery. One of the ewes (pregnant 46 days) was fed only one pellet containing 300 μc. of I^{131} at 20 hours prior to surgery. All ewes were maintained on a diet low in iodine content to insure maximum uptake of the I^{131} (1).

The thyroid gland of each ewe was monitored externally with an ionization chamber (11) at the time of surgery. Blood samples were taken for radiochemical analysis.

Fetuses were autopsied immediately upon removal from the dam. Thyroid glands at these prenatal ages appeared as small, well-defined masses in all cases. They were removed from the fetus under a low-power dissecting microscope by means of glass needles. One thyroid lobe was placed in Bouin's fluid for routine histological and autoradiographic preparations. Digested aliquots of the opposite lobe were plated and radioactivity determined by means of a thin mica end-window Geiger-Müller counter. The autoradiographic methods were similar to those described by Evans (7).

RESULTS

The ewes showed an average uptake of radioiodine of 42 per cent of the administered dose during the time interval of the experiment. This is in accord with results of Bustad *et al.* (1) for normal sheep.

Radioiodine was first detected in the fetal thyroid gland on the 50th day of gestation (Table 1).

Autoradiograms demonstrating uptake of I^{131} in fetal thyroids are shown in Figure 1. The 46–48 day thyroid glands show only background radiation patterns. In the 50-day-old gland there is a uniform distribution of I^{131} throughout the parenchyma of the gland. Focal accumulation of radioiodine is observed in the thyroid of a 52-day-old fetus, reflecting beginning follicle formation at this time. This tendency to focal accumulation increased subsequently as shown in autoradiograms of fetal thyroids from 58, 60, and 70-day-old fetuses.

Histologically, the thyroid glands of the 46, 48, and 50-day fetuses showed cord-like structures of epithelial cells enclosing a rich capillary plexus. The structural pattern of the glands was loose, in that the epithelial cells were often separated from each other and from the capillary walls by a fluid-appearing matrix. No follicles were observed by

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[1] This paper is based on work performed under Contract No. W-31-109-Eng-52 for the Atomic Energy Commission.

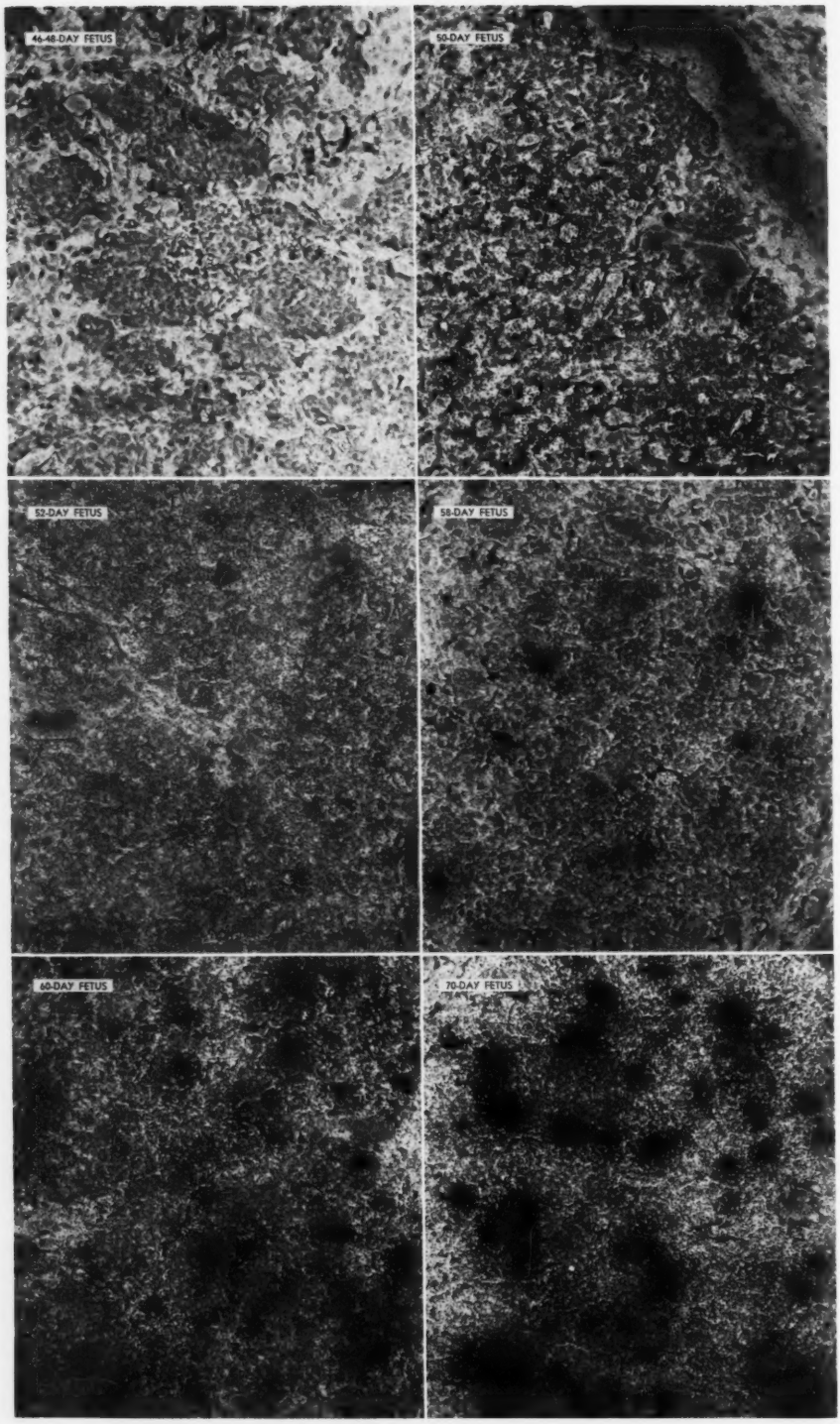

FIG. 1. Photomicrographs (85X) of autoradiograms demonstrating beginning thyroid uptake of I^{131}. (All were exposed 20 days with the exception of the 70-day autogram which was exposed 10 days).

TABLE 1. PER CENT OF MATERNAL DOSE OF I^{131} IN FETAL THYROID

	Days in gestation						
	46	48	50	52	58	60	70
Per cent in Fetal Thyroid	0	0	0.0001	0.04	0.7	0.26	0.44

the 50th day, although their appearance was simulated by epithelial cells surrounding capillaries. In the thyroid tissue of the 52-day fetus a few small but distinct follicles were evident and were accompanied by a compact general arrangement of epithelial tissue. The follicles were limited in number and confined to the periphery in glands from the 52 to 60-day-old fetuses. The 70-day-old fetal thyroids showed many follicles filled with colloid throughout the gland.

DISCUSSION

The simultaneous investigation of I^{131} uptake by autoradiographic, radioanalytical, and histologic methods suggests that collection of iodine by the fetal sheep thyroid gland starts at 50 days of fetal development.

It is concluded that the thyroid begins functioning at the beginning of the second trimester of pregnancy (145-day average gestation period for sheep). This corresponds favorably with the findings of Chapman (8) and Hodges (12) who described beginning I^{131} uptake by the fetal thyroid in man at this period. Beginning function at 50–52 days of gestation is substantially earlier initial function than during the third month reported by Fenger (9) and Hogben (10) who published their work long before radioiodine was available.

SUMMARY

Initial thyroid uptake of I^{121} was demonstrated in sheep fetuses on the 50th day of gestation, using radioanalytical and autoradiographic methods. The formation of thyroid follicles was first observed histologically on the 52nd day of gestation.

The onset of iodine uptake at the beginning of the second trimester of pregnancy in sheep corresponds to the reported time of earliest I^{131} uptake in the human thyroid and occurs substantially earlier than previously reported for sheep.

Acknowledgments

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THE ENDOCRINE SOCIETY

1957 Annual Meeting

The Thirty-ninth Annual Meeting of The Endocrine Society will be held in the Hotel New Yorker, New York, New York, Thursday, Friday, and Saturday, May 30, 31, June 1, 1957.

The Committee on Local Arrangements is Dr. Rulon W. Rawson as Chairman with Drs. Earl T. Engle, Joseph W. Jailer, Warren O. Nelson, and Martin Sonenberg as members of the Committee.

All Scientific Sessions will be held in the Hotel New Yorker. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 31, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Make reservations directly with the New Yorker, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Eleanor Venning, Royal Victoria Hospital, Montreal, Quebec, Canada, not later than February 1, 1957. *It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that these will be presented at the meeting*—in order that they may be of reference value and suitable for printing in the program and Journals of the Society. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be given after his name.
   Line 3. Institution of origin and city in which institution is located.

3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter perfect, since there will be no opportunity for proof reading by authors.

1957 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Medal of The Endocrine Society

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. Dr. Carl R. Moore was the recipient in 1955 and Dr. Frederick L. Hisaw in 1956.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; 1949—Dr. George Sayers; 1950—Dr. Oscar M. Hechter; 1951—Dr. Albert Segaloff; 1952—Dr. Seymour Lieberman; 1953—Dr. Sidney Roberts and Dr. Clara M. Szego (Mrs. Roberts); 1954—Dr. I. N. Rosenberg; 1955—Dr. Jack Gross; 1956—Dr. Alfred M. Bongiovanni. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-four months of the date of the award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2.500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts

in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and The Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually for each individual and shall be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Medal of the Endocrine Society; the Ciba Award; the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *November 1* each year.

Proposals for appointment as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *November 1, 1956.*

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1, 1956.